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INDEX

VOL 88 B FASC 1-6 1980

<i>Aasjord Per</i>	47	<i>Hertz Jesper B</i>	143
<i>Aasjord Per</i>	53	<i>Hertz Jesper B</i>	237
<i>Aasted Bent</i>	323	<i>Hertz Jesper B</i>	243
<i>Aho Ruttia</i>	79	<i>Hertz Jesper B</i>	253
<i>Andersen Bjørn Mari</i>	231	<i>Hoiby E Arne</i>	311
<i>Andersen Hans Kerzel</i>	1	<i>Hoiby Niels</i>	125
<i>Andersen Paul</i>	1	<i>Hoiby Niels</i>	143
<i>Assev S</i>	61	<i>Hoiby Niels</i>	237
<i>Berdal Bjørn Peter</i>	65	<i>Hoiby Niels</i>	243
<i>Berdal Bjørn Peter</i>	89	<i>Hoiby Niels</i>	253
<i>Birch Andersen A</i>	151	<i>Hofstad Tor</i>	115
<i>Birch Andersen A</i>	161	<i>Hovelius Birgitta</i>	225
<i>Brorson John Erik</i>	193	<i>Hutchison W M</i>	151
<i>Burova Larissa A</i>	199	<i>Hutchison W M</i>	161
<i>Christensen Poul</i>	199	<i>Iversen Ole-Jan</i>	329
<i>Christensen Poul</i>	299	<i>Jantzen Erik</i>	89
<i>Danielsson Dan</i>	17	<i>Jarmund T</i>	95
<i>Danielsson Dan</i>	27	<i>Jensen Klaus</i>	143
<i>Danielsson Dan</i>	39	<i>Jensen Klaus</i>	253
<i>Danielsson Dan</i>	207	<i>Jørgensen N H Feilberg</i>	249
<i>Degre Miklos</i>	177	<i>Jonsson Anders</i>	199
<i>Degre Miklos</i>	219	<i>Kamme Carl</i>	261
<i>Eggset Guri</i>	329	<i>Kapperud Georg</i>	65
<i>Eliasson Ingvar</i>	281	<i>Kapperud Georg</i>	287
<i>Eliasson Ingvar</i>	299	<i>Kapperud Georg</i>	293
<i>Espersen F</i>	237	<i>Kapperud Georg</i>	303
<i>Espersen F</i>	243	<i>Kjellander Jan</i>	207
<i>Fand Adel F</i>	307	<i>Klemm P</i>	347
<i>Ferencz A</i>	347	<i>Kosunen Timo U</i>	207
<i>Ferguson D J P</i>	151	<i>Larsen Jens L</i>	307
<i>Ferguson D J P</i>	161	<i>Leerhoy J</i>	349
<i>Gipperi Hans</i>	277	<i>Lillehoy E B</i>	133
<i>Goransson B</i>	133	<i>Mårdh Per Anders</i>	11
<i>Granfors Kaisa</i>	139	<i>Mårdh Per Anders</i>	57
<i>Granroos Paul</i>	139	<i>Mårdh Per-Anders</i>	107
<i>Grov Arne</i>	47	<i>Mårdh Per Anders</i>	225
<i>Grov Arne</i>	53	<i>Maki Markku</i>	139
<i>Grov Arne</i>	121	<i>Mæland Johan A</i>	329
<i>Grubb R</i>	199	<i>Mansa Bendt</i>	143
<i>Gutschik Erno</i>	269	<i>Mansa Bendt</i>	253
<i>Haaheim Lars R</i>	333	<i>Melby Kjetil</i>	103
<i>Haaheim Lars R</i>	341	<i>Melsen Flemming</i>	225
<i>Haaheim Lars R</i>	351	<i>Midtvedt Tore</i>	103
<i>Haukenes Gunnar</i>	85	<i>Müller E</i>	169
<i>Helsingstrand E</i>	169	<i>Møller Birger R</i>	107
<i>Heron I</i>	249	<i>Møller Birger R</i>	225

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Bacteria quantification operation wounds pigs	71	Diarrhoea gastroenteritis <i>Yersinia enterocolitica</i> ELISA	139
Bactericidal test streptococci M protein human IgG Fe anti human Ig Gm markers	199	DNA polymerase hepatitis B trisodium phosphonoformate (PFA)	169
Bacteriophage typing <i>Pseudomonas aeruginosa</i> epidemiological markers sero grouping cystic fibrosis cross infection	125	DNA polymerase inhibitor hepatitis B phosphonoformic acid (PFA) woodchuck hepatitis virus	183
Bacteriuria urinary tract infection pyuria urine sediment	273	DNA small intestinal mucosa	347
Beta lactamase <i>Haemophilus influenzae</i> biotypes	261	Electro immuno assay streptococci Lancefield extract M protein quantitation	299
Biotypes <i>Haemophilus influenzae</i> beta lactamase	261	Electron microscopy antibodies staphylococcal lipoteichoic acid immunoperoxidase	53
Blood culture media <i>Neisseria meningitidis</i>	311	Electron microscopy staphylococcal lipoteichoic acid immunoperoxidase	47
<i>Branhamella catarrhalis</i> antigens serological identification protein A	281	ELISA cytomegalovirus complement fixation	1
Campylobacter antigens Campylobacter serology co-agglutination immunoelectrophoresis	207	ELISA rubella routine diagnosis HI	349
Carriers healthy <i>Yersinia enterocolitica</i> humans	303	ELISA <i>Yersinia enterocolitica</i> diarrhoea gastroenteritis	139
<i>Chlamydia trachomatis</i> Crohn's disease serum antibodies lymphogranuloma venereum TRIC	57	Endotoxin free <i>Neisseria meningitidis</i> antibodies	231
<i>Chlamydia trachomatis</i> salpingitis experimental infection	107	Enterobacteriaceae <i>Salmonella typhi</i> crossed immunoelectrophoresis cross reaction	243
Chloramphenicol resistance antibiotic sensitivity <i>Haemophilus influenzae</i>	193	Enterotoxin <i>Clostridium perfringens</i> membrane leakage	95
Citronin ochratoxin A <i>Aspergillus Penicillium</i> barley	133	Enterotoxin <i>Yersinia enterocolitica</i>	63
Classification <i>Neisseria gonorrhoeae</i> co-agglutination immunoelectrophoresis serology	17	Enterotoxin <i>Yersinia enterocolitica</i>	287
Classification <i>Neisseria gonorrhoeae</i> co-agglutination serology	27	Enzyme sensitivity <i>Pseudomonas aeruginosa</i> Common Antigen molecular weight sedimentation analysis amino acid analysis	253
<i>Clostridium perfringens</i> enterotoxin membrane leakage	95	Epidemiological markers <i>Pseudomonas aeruginosa</i> sero grouping bacteriophage typing cystic fibrosis cross infection	125
Co agglutination Campylobacter serology immunoelectrophoresis Campylobacter antigens	207	<i>Escherichia coli</i> enteropathogenic antibiotic sensitivity mecillinam trimethoprim sulfamethoxazole	264
Co agglutination <i>Neisseria gonorrhoeae</i> immunoelectrophoresis serology	39	<i>Escherichia coli</i> phagocytosis antibodies human polymorphonuclear cells	103
Co agglutination <i>Neisseria gonorrhoeae</i> immunoelectrophoresis serology classification	17	<i>Escherichia coli</i> plasmid coded fimbriae human peripheral lymphocytes adherence	249
Co-agglutination <i>Neisseria gonorrhoeae</i> serology classification	27	<i>Escherichia coli</i> resistance types transfer of amplicillin resistance	317
Common Antigen <i>Pseudomonas aeruginosa</i> isolation Na ₂ SO ₄ precipitation gel filtration crossed immunoelectrophoresis	143	Experimental endocarditis rabbits <i>Serratia marcescens</i> proteolytic capacity serum bacterial activity indwelling catheter	269
Common Antigen <i>Pseudomonas aeruginosa</i> molecular weight sedimentation analysis amino acid analysis enzyme sensitivity	253	Fatty acid composition bacteria gas chromatography taxonomy	89
Antibodies lymphogranuloma venereum TRIC	69	Fatty acid composition <i>Fusobacterium</i> lipopolysaccharide sugar composition	115
Cross infection <i>Pseudomonas aeruginosa</i> epidemiological markers sero grouping bacteriophage typing cystic fibrosis	57	<i>Fusobacterium</i> lipopolysaccharide sugar composition fatty acid composition	115
Cystic fibrosis <i>Pseudomonas aeruginosa</i> epidemiological markers sero-grouping bacteriophage typing cross infection	125	Gas chromatography fatty acid composition bacteria taxonomy	89
Cystic fibrosis	125	Gastroenteritis diarrhoea <i>Yersinia enterocolitica</i> ELISA	139
Diagnosis	61	Gel filtration Common Antigen <i>Pseudomonas aeruginosa</i> isolation Na ₂ SO ₄ precipitation crossed immunoelectrophoresis	143
Diagnosis medium fungal dermatophytes fungal	79	Gm markers streptococci M protein bactericidal test, human IgG Fe anti human Ig	199
	19	Haemagglutination indirect passive haemagglutination rubella	85
		Haemagglutination inhibition rubella routine diagnosis ELISA	349

Møller Susanne	269	Sandsen Per			
Mogensen H H	237	Saron Israel			
Norberg Astrid	277	Schalen Claus			
Norberg Bo	277	Schalén Claus			
Nordenfelt E	169	Schalen Claus			
Nordenfelt E	183	Schuld G C			
Norwood Robert S	269	Sihoni A			
Nystrom Per Olof	71	Siloni A			
Öberg B	169	Sim J Chr			
Oeding Per	121	Sim J Chr			
Orskov F	347	Skaug Nils			
Orskov I	347	Skjalkvåg R			
Olling Sante	269	Sugaard Per			
Omland Tor	65	Solberg Otto			
Omland Tor	89	Soltesz Laszlo I			
Osland Arve	121	Sompolinsky David			
Pedersen Vibeke Barkholt	253	Sompolinsky David			
Ramstorp Goran	299	Svensson Maj-Lis			
Rasmussen Ernst	69	Svensson Maj Lis			
Roe Sissel F	329	Thorén Anders			
Rolla G	61	Tolleshaug H			
Rollag Jr Halvor	177	Ursing Bo			
Rosendal Kirsten	125	Vesjarud G			
Samra Zemira	143	Vestorp M			
Samra Zemira	253	Vesikari Timo			
Samuelsson Gunilla	199	Werner Barbara			
Sandgren Eva	57	Zachrisson Gunilla			
Sandstrom Eric	17				
Sandstrom Eric	27	A See Ae	Æ See Ae	Ö See Oe	
Sandstrom Eric	39	Ø See Oe	Å See Aa		

Aleutian disease virus	323	trachomatis	lymphogranuloma	venereum	
Amino acid analysis		TRIC			
Common Antigen		Antibodies	staphylococcal	lipoteichoic acid	immu
molecular weight sedi			noperoxidase	electron microscopy	
mantation analysis	253	Antibodies	strain specific	influenza	haemaggluti
enzyme sensitivity			nin cross reactive	antibodies	antigenic vari
Ampicillin resistance			ants		
transfer of resistance types	317				
<i>Escherichia coli</i>					
Antibiotic sensitivity					
enteropathogenic <i>E. coli</i>					
meccillinam trimethoprim sulfamethoxazole	265	Antibodies	strain specific	influenza	haemaggluti
Antibiotic sensitivity			nin cross reactive	antibodies	biological acti
<i>Haemophilus influenzae</i>			vity		
chloramphenicol resistance	193				
Antibiotic sensitivity testing					
<i>Vibrio alginolyticus</i> in					
<i>vitro</i>	307	Antibody	monoclonal	influenza	antigenic mutant
Antibiotics		human sera			
<i>Neisseria meningitidis</i> free endotoxin	231	Antigenic mutant	influenza	monoclonal antibody	
Antibiotics		human sera			
phagocytosis human polymorphonuc					
lear cells <i>E. coli</i>	103	Antigenic	variants	influenza	haemagglutinin
Antibodies			strain specific	antibodies	cross reactive anti
cross reactive influenza haemaggluti			bodies		
nin strain specific antibodies antigenic vari	341	Antigens	<i>Brucella melitensis</i>	serological	
ants			identification protein A		
Antibodies					
cross reactive influenza haemaggluti					
nin strain specific antibodies biological acti					
vity	335	Anti human Ig	streptococci M protein	bacterici	
Antibodies		dal test human IgG	Fe Gm markers		
precipitating crossed immunoelectro		<i>Aspergillus Penicillium</i>	ochratoxin A	citrinin	
phoresis <i>Salmonella typhi</i> sero diagnosis ty		barley			
phoid	237	Bacteria	fatty acid composition	gas chromat	
Antibodies			graphy	taxonomy	
serum Crohn's disease <i>Chlamydia</i>					

Bacteria quantification operation wounds pigs	71	Diarrhoea gastroenteritis <i>Yersinia enterocolitica</i> ELISA	139
Bactericidal test streptococci M protein human IgG Fc anti human Ig Gm markers	199	DNA polymerase hepatitis B triiodium phospho noformate (TPFA)	169
Bacteriophage typing <i>Pseudomonas aeruginosa</i> epidemiological markers sero grouping cystic fibrosis cross infection	125	DNA polymerase inhibitor hepatitis B phospho noformic acid (PFA) woodchuck hepatitis virus	183
Bacteriuria urinary tract infection psycia urine sediment	277	DNA small intestinal mucus	347
Beta lactamase <i>Haemophilus influenzae</i> ae biotypes	261	Electroimmuno assay streptococci Lancefield strains M protein quantitation	299
Biotypes <i>Haemophilus influenzae</i> ae beta lactamase	261	Electron microscopy antibodies staphylococcal lipoteichoic acid immunoperoxidase	53
Blood culture media <i>Neisseria meningitidis</i>	341	Electron microscopy staphylococcal lipoteichoic acid immunoperoxidase	47
<i>Branhamella catarrhalis</i> antigens serological identification protein A	281	" "	349
Campylobacter antigens Campylobacter serology co-agglutination immunoelectrophoresis	207	Endotoxin free <i>Neisseria meningitidis</i> antibiotics	139
Careers healthy <i>Yersinia enterocolitica</i> humans	303	Enterobacteriaceae <i>Salmonella typhi</i> crossed immuno electrophoresis cross reaction	243
<i>Chlamydia trachomatis</i> Crohn's disease serum antibodies lymphogranuloma venereum TRIC	57	Enterotoxin <i>Clostridium perfringens</i> membrane leakage	95
<i>Chlamydia trachomatis</i> salpingitis experimental infection	107	Enterotoxin <i>Yersinia enterocolitica</i>	65
Chloramphenicol resistance antibiotic sensitivity <i>Haemophilus influenzae</i>	193	Enterotoxin <i>Yersinia enterocolitica</i>	287
Citronin, ochratoxin A <i>Aspergillus Penicillium</i> barley	133	Enzyme sensitivity <i>Pseudomonas aeruginosa</i> Common Antigen molecular weight, sedimentation analysis amino acid analysis	253
Classification <i>Neisseria gonorrhoeae</i> co agglutination immuno electrophoresis serology	17	Epidemiological markers <i>Pseudomonas aeruginosa</i> sero grouping bacteriophage typing cystic fibrosis cross infection	125
Classification <i>Neisseria gonorrhoeae</i> co agglutination serology	27	<i>Escherichia coli</i> enteropathogenic ambigious sensitivity mecillinam trimethoprim sulfamethoxazole	265
<i>Clostridium perfringens</i> enterotoxin membrane leakage	95	<i>Escherichia coli</i> phagocytosis antibiotics human polymorphonuclear cells	103
Co-agglutination Campylobacter serology immuno electrophoresis Campylobacter antigens	207	<i>Escherichia coli</i> plasmid coded fimbriae human peripheral lymphocytes adherence	249
Co-agglutination <i>Neisseria gonorrhoeae</i> immuno electrophoresis serology	39	<i>Escherichia coli</i> resistance types transfer of ampilicin resistance	317
Co-agglutination <i>Neisseria gonorrhoeae</i> immuno electrophoresis serology classification	17	Experimental endocarditis rabbits <i>Serratia marcescens</i> proteolytic capacity serum bacterial activity indwelling catheter	269
Co-agglutination <i>Neisseria gonorrhoeae</i> serology classification	27	Fatty acid composition bacteria gas chromatography taxonomy	89
Common Antigen <i>Pseudomonas aeruginosa</i> isolates	143	Fatty acid composition <i>Fusobacterium</i> lipopoly saccharide sugar composition	115
" "	253	" " " "	115
Complement fixation cytomegalovirus ELISA	1	" " " "	89
Cover slips safe pathogenic microorganisms	69	Gastroenteritis diarrhoea <i>Yersinia enterocolitica</i> ELISA	139
Crohn's disease <i>Chlamydia trachomatis</i> serum antibodies lymphogranuloma venereum TRIC	57	Gel filtration Common Antigen <i>Pseudomonas aeruginosa</i> isolation Na ₂ SO ₄ precipitation crossed immuno electrophoresis	143
Cross infection <i>Pseudomonas aeruginosa</i> epidemiological markers sero grouping bacteriophage typing cystic fibrosis	125	Gm markers streptococci M protein bactericidal test human IgG Fc anti human Ig	199
Cystic fibrosis <i>Pseudomonas aeruginosa</i> epidemiological markers sero grouping bacteriophage typing	125	Haemagglutination indirect passive haemagglutination rubella	85
" " " "	61	Haemagglutination inhibition rubella routine diagnosis ELISA	349
" " " "	79		
Diagnostic medium fungal diagnostic medium fungal zoonosis	79		
Diagnostic medium fungal dermatophytes fungal zoonosis	79		

Haemagglutination passive indirect haemagglutination rubella		sition fatty acid composition	
Haemagglutinin influenza strain specific antibodies cross reactive antibodies antigenic variants	85	Lymphocytes human peripheral <i>E coli</i> plasmid coded fimbriae adherence	
Haemagglutinin influenza strain specific antibodies cross reactive antibodies biological activity	341	Lymphogranuloma venereum Crohn's disease <i>Chlamydia trachomatis</i> serum antibodies	
<i>Haemophilus influenzae</i> antibiotic sensitivity chloramphenicol resistance	335	TRIC	
<i>Haemophilus influenzae</i> biotypes beta lactamase	193	Macrogamete <i>Isospora felis</i> macrogametogenesis ultrastructure cat	
HeLa cells <i>Yersinia enterocolitica</i> invasiveness	261	Macrogametogenesis <i>Isospora felis</i> macrogamete ultrastructure cat	
Hepatitis B DNA polymerase inhibitor phosphonoformic acid (PFA) woodchuck hepatitis virus	293	Macrophages alveolar interferon phagocytosis Sendai virus	
Hepatitis B DNA polymerase trisodium phosphonoformate (PFA)	183	Mecillinam enteropathogenic <i>E coli</i> trimethoprim sulfamethoxazole antibiotic sensitivity	
Identification serological <i>Branhamella catarrhalis</i> antigens protein A	169	Membrane antigens <i>Neisseria gonorrhoeae</i> periodate resistance	
IgG Fc human streptococci M protein bactericidal test anti human Ig Gm markers	281	Membrane leakage enterotoxin <i>Clostridium perfringens</i>	
Immunochemistry <i>Staphylococcus simulans</i> teichoic acid	199	Microgametogenesis <i>Isospora felis</i> ultrastructure microgamont cat	
Immunoelectrophoresis <i>Campylobacter</i> serology co agglutination <i>Campylobacter</i> antigens	121	Microgamont <i>Isospora felis</i> ultrastructure microgametogenesis cat	
Immunoelectrophoresis crossed Common Antigen <i>Pseudomonas aeruginosa</i> isolation Na ₂ SO ₄ precipitation gel filtration	207	M protein streptococci bactericidal test human IgG Fc anti human Ig Gm markers	
Immunoelectrophoresis crossed cross reaction <i>Salmonella typhi</i> <i>Enterobacteriaceae</i>	143	M protein streptococci Lancefield extract quantitation electro immuno assay	
Immunoelectrophoresis crossed precipitating antibodies <i>Salmonella typhi</i> sero-diagnosis typhoid	243	Na ₂ SO ₄ precipitation Common Antigen <i>Pseudomonas aeruginosa</i> isolation gel filtration crossed immunoelectrophoresis	
Immunoelectrophoresis <i>Neisseria gonorrhoeae</i> co agglutination serology	237	<i>Neisseria gonorrhoeae</i> co agglutination immunoelectrophoresis serology	
Immunoelectrophoresis <i>Neisseria gonorrhoeae</i> co agglutination serology classification	39	<i>Neisseria gonorrhoeae</i> co agglutination immunoelectrophoresis serology classification	
Immunoperoxidase antibodies staphylococcal lipoteichoic acid electron microscopy	17	<i>Neisseria gonorrhoeae</i> co agglutination serology classification	
Immunoperoxidase staphylococcal lipoteichoic acid electron microscopy	53	<i>Neisseria meningitidis</i> membrane antigens periodate resistance	
Influenza antigenic mutant monoclonal antibody human sera	47	<i>Neisseria meningitidis</i> blood culture media	
Influenza haemagglutinin strain specific antibodies cross reactive antibodies antigenic variants	351	<i>Neisseria meningitidis</i> free endotoxin antibiotics	
Influenza haemagglutinin strain specific antibodies cross reactive antibodies biological activity	341	Ochratoxin A citrinin <i>Aspergillus Penicillium</i>	
Interferon murine sarcoma osteosarcoma tissue culture	335	barley	
Interferon phagocytosis Sendai virus alveolar macrophages	219	Operation wounds bacteria quantification pigs	
Invasiveness <i>Yersinia enterocolitica</i> HeLa cells	177	Osteosarcoma murine sarcoma interferon tissue culture	
<i>Isospora felis</i> macrogametogenesis macrogamete ultrastructure cat	293	Parvo viridae aleutian disease virus plasmocytosis	
<i>Isospora felis</i> ultrastructure microgametogenesis microgamont cat	161	<i>Penicillium Aspergillus</i> ochratoxin A citrinin	
Lancefield extract streptococci M protein quantitation electro immuno assay	151	barley	
Lipopolysaccharide <i>Fusobacterium</i> sugar composition	299	Periodate resistance membrane antigens <i>Neisseria gonorrhoeae</i>	
		Phagocytosis antibiotics human polymorphonuclear cells <i>E coli</i>	
		Phagocytosis interferon Sendai virus alveolar macrophages	
		Phosphonoformic acid (PFA) hepatitis B DNA polymerase inhibitor woodchuck hepatitis virus	
		Plasmid coded fimbriae <i>E coli</i> human peripheral lymphocytes adherence	
		Plasmocytosis aleutian disease virus parvo viridae	
		Polymorphonuclear cells human phagocytosis antibiotics <i>E coli</i>	

myxin resistance <i>Proteus Providencia Serratia</i> tetracycline resistance trehalose fermentation	185	Small intestinal mucus DNA	347
strain A <i>Branhamella catarrhalis</i> antigenic serological identification	281	Staphylococcal lipoteichoic acid antibodies immunoperoxidase electron microscopy	53
strains <i>Providencia Serratia</i> polymyxin resistance tetracycline resistance trehalose fermentation	185	Staphylococcal lipoteichoic acid immunoperoxidase electron microscopy	47
<i>Providencia Proteus Serratia</i> polymyxin resistance tetracycline resistance trehalose fermentation	185	<i>Staphylococcus saprophyticus</i> experimental pyelonephritis grivet monkeys	225
<i>Pseudomonas aeruginosa</i> Common Antigen isolation Na_2SO_4 precipitation gel filtration crossed immunoelectrophoresis	143	<i>Staphylococcus simulans</i> teichoic acid immunchemistry	121
<i>Pseudomonas aeruginosa</i> Common Antigen molecular weight, sedimentation analysis amino acid analysis enzyme sensitivity	253	Stools <i>Yersinia enterocolitica</i> selective medium sodium oxalate diagnosis	11
<i>Pseudomonas aeruginosa</i> epidemiological markers sero grouping bacteriophage typing cystic fibrosis cross infection	125	Streptococci Lancefield extract M protein quantitation electro-immuno assay	299
pyelonephritis experimental <i>Staphylococcus saprophyticus</i> grivet monkeys	225	Streptococci M protein bactericidal test human IgG Fc anti human Ig Gm markers	199
pyuria urinary tract infection bacteriuria urine sediment	277	Sucrose substitutes xylitol dental caries	61
Resistance types <i>Escherichia coli</i> transfer of ampicillin resistance	317	Sugar composition <i>Fusobacterium lipopolysaccharide</i> fatty acid composition	115
Rubella indirect haemagglutination passive haemagglutination	85	Taxonomy bacteria fatty acid composition gas chromatography	89
Rubella routine diagnosis ELISA HI	349	Teichoic acid <i>Staphylococcus simulans</i> , immunchemistry	121
Safe cover slips pathogenic microorganisms	69	Tetracycline resistance <i>Proteus Providencia Serratia</i> polymyxin resistance trehalose fermentation	185
	243	Tetracycline sensitivity <i>Serratia</i>	189
		Tissue culture murine sarcoma osteosarcoma interferon	219
		Trehalose fermentation <i>Proteus Providencia Serratia</i> polymyxin resistance tetracycline resistance	185
		TRIC Crohn's disease <i>Chlamydia trachomatis</i> serum antibodies lymphogranuloma venereum	57
		Typhoid <i>Salmonella typhi</i> crossed immunoelectrophoresis precipitating antibodies serodiagnosis	237
		Trimethoprim sulfamethoxazole enteropathogenic <i>E. coli</i> mecillinam antibiotic sensitivity	265
		Trisodium phosphonoformate (PFA) hepatitis B DNA polymerase	169
		Ultastructure <i>Isospora felis</i> macrogametogenesis macrogamete cat	161
		Ultastructure <i>Isospora felis</i> microgametogenesis microgamete cat	151
		Urinary tract infection bacteriuria pyuria urine sediment	277
		Urine sediment, urinary tract infection bacteriuria pyuria	277
		<i>Vibrio alginolyticus</i> antibiotic sensitivity testing in vitro	307
		Woodchuck hepatitis virus hepatitis B DNA polymerase inhibitor phosphonoformic acid (PFA)	183
		Xylitol sucrose substitutes dental caries	61
		<i>Yersinia enterocolitica</i> ELISA diarrhoea gastroenteritis	139
		<i>Yersinia enterocolitica</i> enterotoxin	65
			287
			303
			293

<i>Yersinia enterocolitica</i> : selective medium, sodium oxalate, stools, diagnosis	11	Zoonosis, fungal, fungal diagnostic medium, dermatophytes
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SUPPLEMENTS

Supplement 272	<i>Linell Folke, Ljungberg Otto and Andersson Ingvar</i> Breast Carcinoma Aspects of early stages, progression and related problems Pp 233 1980 (Section A)
Supplement 273	<i>Nielsen Henrik</i> Circulating Immune Complexes Properties, Methods for Detection and Disease Models Pp 56 1980 (Section C)

ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETERMINATION OF IgG ANTIBODIES TO HUMAN CYTOMEGALOVIRUS

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A solid phase enzyme linked immunosorbent assay (ELISA) for determination of IgG antibodies to
cytomegalovirus (CMV) is described. The assay uses purified CMV and extracts of CMV infected cells
as antigen. Antigens were desiccated onto the bottom surface of polystyrene microcuvettes. The
antibodies bound to the antigens were assayed by anti IgG alkaline phosphatase conjugate followed by
addition of the enzyme substrate. Titration curves have been obtained from the sera of 35 blood donors

and of 22 sera from CMV infected patients. The ELISA was compared with the complement fixation (CF) test
and with those obtained by complement fixation (CF) test. The ELISA was of value particularly in those sera

which show high reactivity against control antigen. The ELISA technique described is approximately
412 ± 548 times more sensitive than the CF test when purified CMV or extracts of CMV infected
cells respectively are used as antigens. No significant heterotypic rise to CMV was observed by
ELISA in three sets of sera with seroconversion to herpes simplex virus. The ELISA technique gives
objective results is easily performed and may be adaptable as a routine test both for serological
diagnosis of CMV infection and for screening of the general population.

Key words: CMV, ELISA, CF.

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Human cytomegalovirus (CMV) is a major viral
cause of birth defects which include mental
retardation, blindness and deafness (26). In the
normal human population, CMV infections are
generally subclinical and the virus remains in a
latent, potentially infectious form. CMV infection

may constitute a serious problem for immunosuppressed
individuals such as recipients of organ transplants
and cancer patients (20).

The accurate diagnosis of CMV infection is of
major concern to medicine. Currently, the methods
used most frequently are based on virus isolation
and detection of antibodies to CMV by the
complement fixation (CF) test, the indirect haemagglutination assay (IHA) (13) or the indirect
fluorescent antibodies assay (IFA) (8). Recently, an
indirect solid phase radioimmunoassay (RIA) has
been developed for measurement of antibodies to
CMV (17). The sensitivity of the RIA accounts for
its widespread growth as an assay technique.
However, this technique possesses certain disadvantages, viz. the short self life of reagents and the

SUPPLEMENTS

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|----------------|---|
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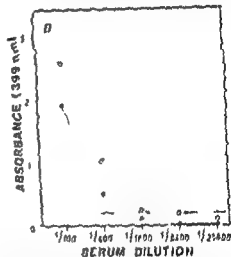
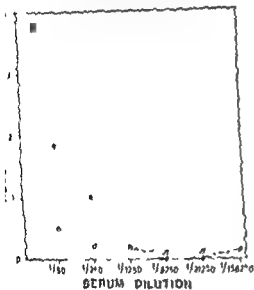
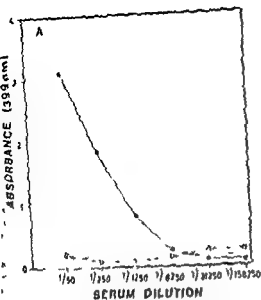
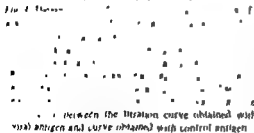


Fig. 2 Titration curve of a positive serum on purified CMV antigen (A) and extracts of CMV infected cell antigen (B) showing high control antigen reactivity giving a false negative result.

Solid lines were tested on CMV antigen
Dotted lines were tested on control antigens



the intersection of the titration curve of the viral antigen with an arithmetic average of the background. Since we are detecting antibodies by an enzymatic reaction we wished to verify our end-point determination by prolonging the reaction time. Table 1 demonstrates this experiment performed with purified CMV as antigen. When absorbance readings (A_{399}) were taken 25 hours after substrate addition, one obtained approximately the same titre as after 43 hours viz 1/9 x 10⁴. With time the absorbance of each serum dilution approached the maximum reading of the EKB apparatus, 9.9 beyond the end point (in this case

1:1000) by ELISA with purified CMV antigen (Fig. 1C).

As already mentioned we have chosen arbitrarily and without cause for the purified CMV antigen as

potential health hazard of the radiation. A promising alternative to RIA appears to be the enzyme linked immunosorbent assay (ELISA) developed recently by a number of investigators (2, 9-11, 27). Recent studies have shown that this method is suitable for antibody assay in diagnostic microbiology (3, 5, 6, 15, 16, 18, 23).

In preliminary studies Voller & Bidwell (27) and Castellano *et al* (7) have described a micro ELISA method for CMV IgG antibodies using commercial CMV antigens and extracts of CMV infected cells. Schmutz *et al* (25) developed ELISA for determination of CMV IgM antibodies by applying nuclei of CMV infected cells as antigen. The present study reports the development of ELISA for detecting CMV IgG antibodies using both purified CMV and extracts of CMV infected human embryonic fibroblasts (HEL) as antigens. We have compared the sensitivity of this CMV IgG ELISA with that of the complement fixation test (CF).

MATERIALS AND METHODS

Sera Eighty one sera were examined including 10 from 5 patients with CMV infection, 6 from 3 patients with herpes simplex virus (HSV) stomatitis and 30 from patients with other viral diseases as well as 35 healthy blood donors. All sera were inactivated at 56 °C for 30 min and stored at -20 °C until use.

Viral antigens Highly purified CMV strain AD 169 was obtained from the supernatant fluids of infected HEL cells by successive zone and density centrifugation as described by Sarov & Abady (24). The infectivity of the viral preparations was determined by the method of Plummer & Benyesh Melnick (22). A virus suspension of 1 OD_{260nm} gave 10⁷-10⁸ PFU/ml. The viral bands were dialysed overnight against phosphate buffered saline (PBS) pH 7.2 and adjusted to 20 µg of protein per ml as determined by Lowry *et al* (19). The purified viral antigens were divided into small aliquots and stored at -70 °C. Control antigen to the purified virus was obtained by processing supernatant fluid from fibroblasts of the same line in the same way as that used to obtain purified CMV.

CMV HEL antigen was produced by infecting cells in Roux bottles with 1 PFU/cell of CMV strain AD 169. After 9-12 days the infected cells were washed with 10 ml of PBS, frozen and thawed 3 times in 3 ml of coating buffer pH 9.6 (27). Cell debris was removed by centrifugation at 300 g for 10 min. The supernatant was divided into small aliquots and stored at -70 °C. Control antigen was prepared in the same way from non infected cells and stored at -70 °C.

ELISA The procedure is a modification of that described previously by Voller & Bidwell (27). Twenty five microlitre drops of the various antigens were air dried overnight at room temperature in polystyrene cuvettes (LKB). The dried antigens were washed 3 times

with phosphate buffered saline pH 7.4 containing 0.05% Tween 20 (PBS T). 0.1 ml of four fold serum dilutions in PBS T was added and the sample duplicate were incubated for 3 hours at room temperature under continuous shaking in a Marius shaker. Cuvettes were washed 3 times with PBS T and 0.1 ml conjugated swine antibodies against human IgG (OR Diagnostica - Finland) was added and incubated overnight with shaking at room temperature. Conjugate was used at dilution 1:400 in PBS T. The day the cuvettes were washed 3 times with PBS T. 0.1 ml of p-nitrophenyl phosphate substrate (Sigma) concentration of 1 mg/ml in 10% diethanolamine buffer pH 9.8 was added. The cuvettes were incubated at room temperature for 1 hour and the reaction stopped by adding 0.5 ml of 0.3 N NaOH. Absorbance at 399 nm was measured in an LKB 7400 calculator spectrophotometer. Within a linear absorbance range apparatus was adjusted to express the absorbance values between 0 and 9.9. Cuvettes containing distilled water were used as reference blanks. Known positive and negative sera were included in each experiment as 6 cuvettes containing only serum diluent instead of serum. An average background adsorption level was determined and this value was subtracted from a sample before plotting the curves.

Other serological methods The complement fixation test (CF) was performed as described previously (1).

RESULTS

Determination of Serum Titre

Fig 1 demonstrates the titration curves of known CMV positive sera tested on purified virus (Fig 1A), extracts of CMV HEL (Fig 1B) and control antigens (dotted lines). For the purified virus we have chosen the intersection of the titration curve using viral antigen with the background line as the titre of the serum. The background line marks the absorbance value at 399 nm (A₃₉₉) obtained with serum diluent on viral antigen instead of serum (average of 6 determinations). When the standard CMV positive serum was titrated in five experiments using two different batches of purified virus the maximum difference in titre was in the two-fold range.

When extracts of CMV HEL (Fig 1B) were used as antigen we took the intersection between the titration curve obtained with viral antigen and the corresponding curve obtained with control HEL antigen as the viral titre.

While most of the sera react only slightly with control HEL antigen (at most the two first dilutions of test sera) we found a false negative result in transplant patient in whose serum high non specific reactivity with control HEL antigen was detected (Fig 2A). This serum has a titre of 16 by CF at

TABLE 2 *CMV Antibody Titre of Paired Sera of Patients with Seroconversion to Herpes Simplex Viruses*

Donors	Age (years)	Date	HSV titre†	CMV ELISA	
				purified CMV	CMV HEL ag
M T	4	17-5-76	4	<100	<100
		9-6-76	32	<100	<100
T T	6	21-5-76	4	<100	<100
		9-6-76	64	<100	<100
S M	5	10-5-76	4	<100	<100
		31-5-76	32	<100	<100

† Determined by complement fixation test (1)

protein per ml of CMV HEL antigen resulted in no significant change in the sensitivity. With lower antigen concentration (10-50 µg protein per ml) the assay sensitivity was decreased and particularly the discrimination between positive and negative sera (data not shown). A concentration of 200 µg protein per ml was chosen for use in further experiments. The highest sensitivity with purified CMV antigen was obtained with the highest concentration of CMV antigen tested (20 µg protein per ml). At lower antigen concentration (5 and 1.25 µg/ml) the assay sensitivity was markedly decreased.

Specificity of the Assay

To exclude the possibility of heterologous cross reactivity between antigens of CMV and herpes simplex virus we used both purified and CMV HEL antigens to test the titres of three paired sera which showed seroconversion to herpes simplex type 1. Table 2 shows that when there is a specific rise in complement fixing (CF) antibodies against herpes simplex virus no corresponding rise was found by ELISA against either purified CMV or extracts of CMV HEL antigens.

TABLE 3 *Comparison of CF and ELISA Titres of Patients with Acute CMV Infections*

Sera No	Clinical diagnosis	Age (years)	Time from first serum (days)	CF	Antibody titres ELISA		Absorbance (399 nm)	
					purified CMV	CMV HEL Ag ¹	purified CMV	CMV HEL ag ¹
13473	Reticulo- erythema	55	0	16	12 500	850	2.0	0.7
13474			21	256	80 685	12 500	3.0	1.9
13746	Renal transplant	46	0	64	9 300	1 800	0.9	1.0
1262			83	256	32 000	6 250	2.6	2.0
2836	Renal transplant	33	0	8	3 375	1 700	0.6	0.7
3009			30	128	16 250	25 000	2.8	2.0
2837	Renal transplant	41	0	8	5 625	4 250	1.2	1.2
3017			36	256	25 000	30 000	2.2	2.6
3001	Renal transplant	33	0	4	50	50	0.1	0.0
3002			13	32	250	250	0.2	0.2

TABLE I Influence of Reaction Time on CMV ELISA Titre Determination on Purified CMV Ag

Reaction time (hours)	Absorbance values (399 nm)							CMV titre
	Serum dilutions	50	250	1,250	6,250	31,250	156,250	
2½		3.10	1.90	0.90	0.25	0.10	0.10	16 × 10
9		7.20	4.30	1.80	0.50	0.12	0.06	18 × 10
19		9.90	7.58	3.48	0.71	0.10	0.03	19 × 10
30		9.90	9.30	4.60	1.06	0.08	0.06	19 × 10
43		9.90	9.90	5.60	1.32	0.10	0.05	19 × 10

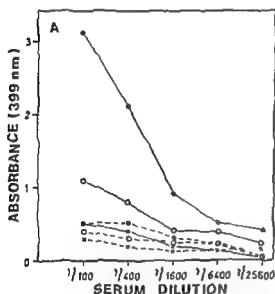
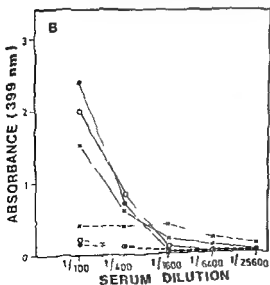


Fig 3 Serum titration curves showing effect of varying concentration of purified CMV (A) and extracts of CMV HEL antigen (B) dried onto microcuvettes
A) Purified CMV antigen ● 20 µg/ml ○ 5 µg/ml
1.25 µg/ml antigen concentration
B) Extracts of CMV HEL antigen (B) ● 800 µg/ml ○ 200 µg/ml X 50 µg/ml
Solid lines, sera tested on CMV antigens
Dotted lines, sera tested on control antigens



dilution 1/31250) there was no net increase in A₃₉₉ value with time, which shows that the endpoint is indeed reached. We have performed measurements similar to those described above on 36 sera which include CMV patients and blood donors, and similar results were obtained. However, it should be noted that prolongation of the reaction time is not suitable for discrimination between low positive and negative sera, since some of the negative sera with time showed a non-specific positive reaction. Confirmation of the viral titre by prolonging the reaction could be performed with CMV HEL antigen also but in that case the reaction time should be prolonged for no more than 24 hours on account of the high rise in background activity.

Determination of Optimal Antigen Concentration

The optimal concentration of various antigens was determined by titration of one positive and one negative serum against known CMV HEL antigen (Fig 3 B) and purified CMV antigen (Fig 3 A). Using an antigen concentration of 200 to 800 µg/ml

TABLE 2 *CMV Antibody Titre of Paired Sera of Patients with Seroconversion to Herpes Simplex Viruses*

Donors	Age (years)	Date	HSV titres ^a	CMV ELISA	
				purified CMV	CMV HEL ag
MT	4	17-5-76	4	<100	<100
		9-6-76	32	<100	<100
TT	6	21-5-76	4	<100	<100
		9-6-76	64	<100	<100
SC	5	10-5-76	4	<100	<100
		31-5-76	32	<100	<100

^a Determined by complement fixation test (1)

protein per ml of CMV HEL antigen resulted in no significant change in the sensitivity. With lower antigen concentration (10-50 µg protein per ml) the assay sensitivity was decreased and particularly the discrimination between positive and negative sera data not shown). A concentration of 200 µg protein per ml was chosen for use in further experiments. The highest sensitivity with purified CMV antigen was obtained with the highest concentration of CMV antigen tested (20 µg protein per ml). At lower antigen concentration (5 and 1.25 µg/ml) the assay sensitivity was markedly decreased.

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TABLE 3 *Comparison of CF and ELISA Titres of Patients with Acute CMV Infections*

Sera No	Clinical diagnosis	Age (years)	Time from first serum (days)	CF	Antibody titres ELISA		Absorbance (399 nm) Dil 1:250	
					purified CMV	CMV HEL Ag ^a	purified CMV	CMV HEL ag ^a
13473	Reticulo sarcoma	57	0	16	12 500	850	1.0	0.7
13481			81	256	80 685	12 500	3.0	1.9
13746	Renal transplant	46	0	64	9 300	1 800	0.9	1.0
2262			85	256	32 000	6 250	2.6	2.0
2836	Renal transplant	33	0	8	3 375	1 700	0.6	0.7
3009			30	128	16 250	25 000	2.8	2.0
2857	Renal transplant	41	0	8	5 625	4 250	1.2	1.2
3023			36	256	25 000	30 000	2.2	2.6
3001	Renal transplant	33	0	4	50	50	0.1	0.0
3002			13	32	250	250	0.2	0.2

^a CMV HEL ag: extracts of CMV HEL cells antigen.

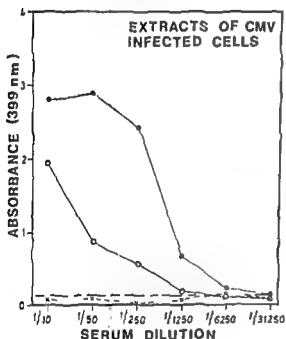
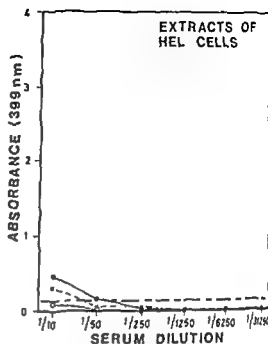
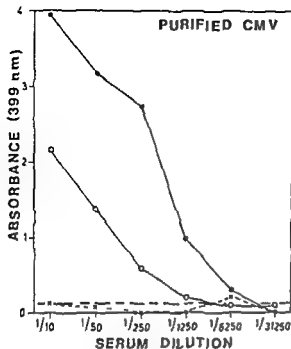


Fig 4 Diagnosis of CMV infection paired sera of a 3 year old renal transplant patient showing a rise in ELISA titre with purified CMV and extracts of CMV and HEL as antigens

Solid lines open symbols first serum
Solid lines solid symbols second serum taken 36 days later
Dotted lines negative sera

patient, a titre of 80 685 was obtained by ELISA compared to 256 by CF. It should be noted that for four of five of our CMV patients a difference in absorption at 399nm between acute and convalescent sera was found at a serum dilution of 1/250 when the reaction was carried out for 1 hour (standard conditions). Thirty sera of 15 patients with unrelated diseases were examined and none showed significant CMV titre differences between acute and convalescent sera by ELISA or by CF.

Blood Donors

A sample of 35 sera of presumably healthy blood donors was evaluated for CMV antibody titre by ELISA and by CF using both purified and CMV HEL antigens.

a) *Negative sera* Eighteen out of these 35 sera were negative by the CF test (titre < 4). All 18 were negative by ELISA (titre < 100) using CMV HEL antigens. These sera had control antigen curves either identical with or slightly higher than viral antigen curves. Fourteen of the 18 were negative by ELISA using purified CMV antigen. However, 4 of the 18 showed a higher reactivity against purified antigen than against control HEL

Patients with CMV Infections

The ELISA results obtained for paired sera of 5 CMV patients (Table 3) were compared with those obtained by CF. Fig 4 illustrates ELISA results obtained for one serum pair from a CMV patient with both purified and CMV HEL antigens. With both antigens a diagnostic titre rise could be demonstrated (purified CMV antigen 5625 to 25 000 CMV HEL antigen 4250 to 30 000).

In both procedures four fold or greater rises in titre were observed for each of the serum pairs (Table 3). The ELISA gave titres much higher than those obtained by CF. For example for one CMV

TABLE 4 Comparison of CF and ELISA Titres of CMV Positive Blood Donors

Sera No	Antibody titres		
	CF	ELISA	
		Purified CMV	CMV HEL Ag
5538	4	2100	6400
5513	8	2600	2000
5512	8	3400	10 000
5519	8	3600	6400
5525	8	5300	5000
5926	8	5500	10 000
5925	8	6400	10 000
5511	16	3800	10 000
5666	16	5100	20 000
5712	16	8000	10 000
5772	16	9700	6400
5517	16	10 000	20 000
5536	16	15 000	4000
5515	32	5000	10 000
5514	32	18 000	6400
5734	64	13 000	20 000
5941	128	13 000	10 000
GMT ^a	15.4	6336	8419

^a Geometric mean titre (GMT)

antigen giving titres of about 1/400. To investigate the possibility that the low titre of about 1/400 obtained above in 4 of these sera represents a non-specific reaction of the sera to the purified virus these sera were re-examined by including a protein supplement consisting of 1% bovine albumin in PBS. The supplement was added to the antigen-treated cuvettes for 1 hour at room temperature and subsequently washed off. Two of these sera became completely negative whereas in the other two no change in titre was obtained.

b) *Positive sera.* Of the sample of 35 sera 17 were found positive by CF test. All 17 were positive both when using purified CMV antigen and when using CMV HEL antigen. Sera which showed low titres of CF also showed low titres by ELISA with purified CMV antigen (Table 4) and vice versa. However the correlation between CF and ELISA was not complete and is less clear when CMV HEL antigen is used in ELISA (Table 4). The average geometric mean titre (GMT) for the CMV positive blood donors by CF is 15.4 whereas by ELISA a GMT 6336 and 8419 was obtained using purified CMV or CMV HEL antigen respectively.

DISCUSSION

The present study demonstrates the applicability of the ELISA system as an additional serological tool for determination of CMV IgG antibodies. Under our experimental conditions both purified and CMV HEL antigens were suitable as antigens (Table 3 Table 4). However a false negative reaction or error in titre determination may occur in those sera which possess very high reactivity against control HEL antigen (Fig. 2). Purified CMV antigen could be of value particularly in those sera which contain high non-specific reactivity against control HEL antigen (Fig. 2 B).

There is no generally accepted formula for determination of serum titre by ELISA. We have chosen the end point titre as the intersection of the titration curve with a background line (the A₃₉₉ value of the serum diluent instead of serum) in the case of purified CMV antigen and the intersection of the titration curve on viral antigen with the corresponding curve on control antigen when crude antigen extracts of CMV infected cells are used. In the RIA systems some investigators defined as a cut-off point, background + 2 (21) or + 3 (4) standard deviations. Other establish a reference line of two times a background defined as the average of 12 replicates of viral antigens and 12 replicates of control antigen (17) and some determine titre as the last point at which there is no longer a ratio of at least 2:1 between counts bound to viral antigen and counts bound to control antigen (12). Since we are detecting antibodies by an enzymatic reaction we could confirm our assumption by prolonging the reaction time (Table 1). With time there is a gradual rise in the absorbance of each serum dilution showing antibody activity but no rise beyond the end point. This experiment indicates that we have a proper method for calculating the ELISA titres.

The ELISA system appears to be specific and no cross reactivity with herpes simplex virus was observed (Table 2). However many more samples of paired sera will have to be examined in order to determine whether the method is completely specific. To examine further the specificity of ELISA serum titrations were obtained for paired sera of 5 CMV patients using purified and CMV HEL antigen (Table 3). The results of ELISA titration showed complete agreement with those obtained by CF. By both methods a significant rise in titre was obtained for each serum pair. The specificity of ELISA test was analysed further on 35 presumably healthy blood donors using both purified and CMV HEL antigens (Table 4). These results were then compared with CF titres. It was found that all the

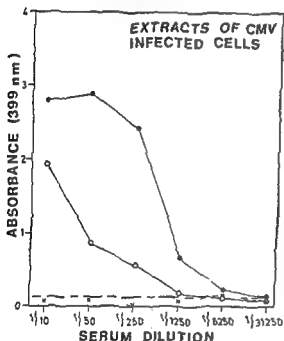
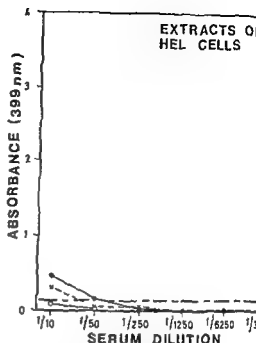
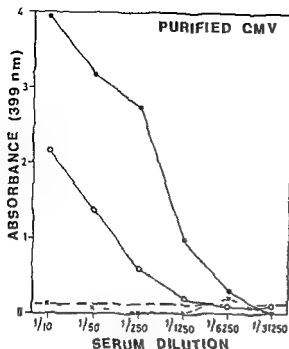


Fig 4 Diagnosis of CMV infection paired sera of a year old renal transplant patient showing a rise in titre with purified CMV and extracts of CMV and as antigens

Solid lines open symbols first serum

Solid lines solid symbols second serum taken 36 later

Dotted lines negative sera

patient a titre of 80 685 was obtained by ELISA compared to 256 by CF. It should be noted that four of five of our CMV patients a difference in absorption at 399nm between acute and convalescent sera was found at a serum dilution of 1 when the reaction was carried out for 1 hour (standard conditions). Thirty sera of 15 patients with unrelated diseases were examined and none showed significant CMV titre differences between acute and convalescent sera by ELISA or by CF.

Patients with CMV Infections

The ELISA results obtained for paired sera of 5 CMV patients (Table 3) were compared with those obtained by CF. Fig 4 illustrates ELISA results obtained for one serum pair from a CMV patient with both purified and CMV HEL antigens. With both antigens a diagnostic titre rise could be demonstrated (purified CMV antigen 5625 to 25 000 CMV HEL antigen 4250 to 30 000).

In both procedures four fold or greater rises in titre were observed for each of the serum pairs (Table 3). The ELISA gave titres much higher than those obtained by CF. For example for one CMV

Blood Donors

A sample of 35 sera of presumably healthy blood donors was evaluated for CMV antibody titre by ELISA and by CF using both purified and CMV HEL antigens.

a) *Negative sera* Eighteen out of these 35 sera were negative by the CF test (titre < 4). All were negative by ELISA (titre < 100) using CMV HEL antigens. These sera had control antibody curves either identical with or slightly higher than viral antigen curves. Fourteen of the 18 were negative by ELISA using purified CMV antigen. However 4 of the 18 showed a higher reactivity against purified antigen than against control HEL

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17 sera which were positive by CF were also positive by ELISA

The sensitivity of the ELISA method is, however, up to 412 or 548 times the sensitivity obtained by the CF test. This determination is based on the assumption that a comparison of geometric mean titre (GMT) can give an approximation of relative sensitivities of 2 techniques. The GMT of the blood CMV positive donors is 15.4 by CF and 6336 and 8419 by ELISA when applying purified and CMV HEL antigens, respectively.

A measure of the reliability of a test is its ability to discriminate between positive and negative sera. With extracts of CMV HEL as antigen, all the 18 sera which were negative by CF (<4) were also found negative by ELISA (<100). Thus, with CMV HEL antigen, the ELISA system discriminates between positive and negative sera. With purified CMV antigen 2 out of these 18 sera were found to be positive by ELISA (1/400) but negative by CF (<4). These latter two sera should thus be considered to be either false positive or as having a very low titre of CMV antibodies (1/400) which could be detected only by purified CMV antigen but not when using CMV HEL antigen. It should be noted that for purified CMV it is difficult to prepare a real control antigen, and the control antigen prepared from supernatant of uninfected HEL does not contain any detectable protein (24).

Although it has been reported that CMV infected cells possess Fc receptors (12, 14) these were not found by ELISA, since the negative sera have either identical titration curves on viral and control antigen or in some cases higher control antigen values. It is possible that our antigen preparations do not contain the Fc receptors, since the CMV HEL antigen consists of an extract of frozen and thawed cells from which cell debris has been removed by centrifugation.

To summarize the present study demonstrates that ELISA CMV IgG can be considered as a potential sensitive test for use both in diagnostic laboratories and for population screening studies.

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AN EFFECTIVE, SELECTIVE MEDIUM FOR *YERSINIA ENTEROCOLITICA* CONTAINING SODIUM OXALATE

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Soltész L V Schalen C & Mårdh P A An effective selective medium for *Yersinia enterocolitica* containing sodium oxalate Acta path microbiol scand Sect B 88 11-16 1980

A medium (»Y« medium) is described which was more efficient for the isolation of *Yersinia enterocolitica* from experimentally infected faecal specimens than desoxycholate-citrate McConkey lactose sucrose urea (LSU) agar and *Yersinia* selective medium (Waters medium). The »Y« medium consists of casein hydrolysate and peptone serving as carbon and energy sources. A high selectivity is achieved by its contents of sodium oxalate and bile salts. The oxalate suppresses growth of gram-negative rods including members of the family *Enterobacteriaceae* and of *Pseudomonas* spp. while the bile salts inhibit growth of gram-positive bacteria. In the few instances coliform rods grew on the »Y« medium they could easily be distinguished by their fermentation of lactose included in the medium and the fact that colonies of organisms were surrounded by an opaque zone of precipitated bile salts. The most optimal condition for the isolation of *Y. enterocolitica* from stools was achieved at incubation of the »Y« medium at 29 °C for 2 days.

Key words: *Yersinia enterocolitica*, selective medium, sodium oxalate, stools, diagnosis.

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During recent years infections with *Yersinia enterocolitica* in Europe particularly with serotypes 3 and 9 (10, 16, 22) and in the US with serotype 8 (16, 20, 22) have been diagnosed by increasing frequency in man. The organism has been associated with acute terminal ileitis, lymphadenitis mesenterica in children, enteritis, septicemia, erythema nodosum and arthritis. It may also be isolated from patients with other diseases.

In the present study we have evaluated the efficiency of the »Y« medium for the isolation of *Y. enterocolitica* from faecal specimens. The medium was compared with desoxycholate-citrate (DC), lactose-sucrose-urea (LSU), MacConkey (MC) agar (10, 17) as on a medium specifically designed for isolation of

this organism viz *Yersinia* selective medium (YS) (19). However, on attempts to recover *Y. enterocolitica* from faecal specimens, differences in the isolation frequency when using these media are noted (10, 11, 19). Highly selective media seem to promote the recovery of *Y. enterocolitica* (11, 19). Incubation of stools at 4 °C for 3 weeks has also been demonstrated to increase the recovery frequency (4, 5, 12, 18, 19).

Studies on nutritional requirements of *Y. enterocolitica* resulting in the composition of a selective differentiating medium (»Y« medium) for this organism are presented. The efficiency of the »Y« medium as compared with other media currently used for the isolation of *Y. enterocolitica* for the recovery of the organism from experimentally infected faecal specimens is also described.

Table 1 Constituents of a New Selective Culture Medium for *Yersinia enterocolitica*

Compound (designation/manufacturers)	Grams/litre distilled water
Bacteriological Peptone (L 37 Oxoid)	15
Casein Hydrolysate (L 41 Oxoid)	5
Lactose (Mallinkrodt)	10
Sodium Oxalate (BDH)	8
Sodium Desoxycholate (6504 Merck)	16
Sodium Chloride (6404 Merck)	5
Bile Salts (L 55 Oxoid)	5
Neutral Red (1369 Merck)	0.08
Bacto Agar (B 140 Dico)	20

RESULTS

Composition of the «Y» Medium

The composition of the «Y» medium is given in Table 1. The use of both peptone and casein hydrolysate as carbon sources was found to be essential. The casein hydrolysate seemed to promote rapid growth while peptone permitted development of large colonies of *Y. enterocolitica* (Table 2).

Selectivity and Recovery Frequency of *Y. enterocolitica* from Experimentally Infected Faecal Specimens on the «Y» Medium and Different Variants Thereof

When inoculating faecal specimens on the «Y» medium, growth of gram positive bacteria was almost completely inhibited by the bile salts. The growth inhibitory effect on gram positive bacteria was clearly demonstrated in the tests using «Y» medium to which bile salts had not been added. Sodium desoxycholate at the concentration used not only suppressed growth of gram positive bacteria but also that of most gram negative rods

Table 3 Recovery of *Yersinia enterocolitica* from Experimentally Infected Faecal Specimens after 2 Days Incubation at 29°C Using Five Different Media. Designation of Media see Text

Medium	Number of stools infected (N)	Reisolation of <i>Y. enterocolitica</i> from no. of stools	
		n	% of N
MC	197	175	88.8
DC	204	184	90.2
YS	116	96	82.7
LSU	142	126	88.7
«Y»	224	223	99.5

including *Proteus* and *Pseudomonas* spp. Occasionally some colonies of gram negative rods other than *Y. enterocolitica* appeared on the «Y» medium. If present these bacteria could easily be differentiated from *Y. enterocolitica* because they produced acid from lactose and/or precipitated the bile salts which resulted in a red and/or opaque zone around the colonies.

In the comparative studies of experimentally infected stools in which dicarboxylic acids other than oxalate viz. 1 ketoglutarate and succinate were used the highest recovery frequency of *Y. enterocolitica* viz. 100% as well as the largest average colony-diameter were found when oxalate was used. In this respect HBA was almost as efficient. When the medium was supplied with sodium 1 ketoglutarate or succinate only 80% and 90% respectively of the tested 40 strains were recovered and the average colony-diameter was smaller (Table 4).

In the comparative studies using increasing amounts of sodium oxalate a concentration of 1%

Table 2 Number of 40 Strains of *Yersinia enterocolitica* Growing on the New Medium Containing Bacteriological Peptone and Casein Hydrolysate 15 and 5 g per Litre Respectively (Y) as Compared to the Same Medium with an Increased Concentration of Bacteriological Peptone 20 g per Litre (Y BP), or of Casein Hydrolysate 20 g per Litre (Y CH) after 2 Days Incubation at 29°C. Diameter of Colonies

Medium	Mean number of colony forming units		Diameter of colonies (mm)	
	24 hours	48 hours	Mean	Range
Y	23	31	2.3	1.3-2.5
Y BP	17	30	2.3	1.2-2.6
Y-CH	22	28	1.7	1.3-1.9

The inoculum used resulted in approximately 30 colony forming units as tested on blood agar plates.

MATERIALS AND METHODS

Test Strains

Altogether 81 strains of *Y. enterocolitica* all of human origin were used in this study. Of the strains 42 were obtained from Prof S Winblad Malmö Sweden while 26 strains came from Dr K Gaarslev Statens Serum institut Copenhagen Denmark. The remaining 13 strains studied were isolated from faecal specimens sent to the Bacteriological Laboratory University Hospital Lund Sweden. These latter strains were identified as *Y. enterocolitica* on the basis of colony morphology gram staining biochemical reactions (see below) which were in principle performed as recommended by Nlehn (11) and agglutination tests using antisera purchased from Statens Bakteriologiska Laboratorium Solna Sweden.

Biochemical Reactivity of Test Strains

All 81 strains formed acid from L-arabinose cellobiose D-fructose D-galactose D-glucose D-mannitol D-mannose sorbitol sucrose and D-trehalose at both 22 °C and 37 °C. They all decarboxylated ornithin but not arginin and lysin. They also hydrolysed urea and 2-nitrophenyl beta-D-galactoside (ONPG) and were positive in the methyl red test. None of the 81 strains fermented adonitol dulcitol inulin lactose raffinose rhamnose or D-xylose. Phenylalanin was not deaminated. Aesculin hydrolysis and H₂S production were never demonstrated. The oxidase reaction was negative for all strains. The Voges-Proskauer test was positive at 22 °C but negative at 37 °C. This was also true for fermentation of maltose. The biochemical tests were read after 2 and 3 days incubation at the two temperatures indicated.

Composition of the 'Y' Medium and Different Tested Variants Thereof

The composition and the suppliers of the constituents of the recommended selective medium for *Y. enterocolitica* are given in Table 1. The following batches of constituents were used: Casein hydrolysate (acid) batch no 158 9783 and 23 711616; Bacteriological peptone batch no 1 816071 and 29 912579; D-(+)-lactose batch no WCHS 86 711-1; sodium oxalate batch no 8 535837; neutral red batch no 6 010861; Bacto agar batch no 644091; and bile salts.

In the comparative experiments described below 40 artificially infected specimens were used (see below). In some of these experiments the 'Y' medium from which bile salts were omitted was used. In other studies the sodium oxalate in the 'Y' medium was replaced by either 1 ketoglutarate (Merck Darmstadt W Germany batch no 6323613) or sodium succinate (Merck batch no 8522485) which both were used in concentrations equimolar to that of oxalate viz 0.06 M. The optimal concentration of sodium oxalate was assessed in experiments using the 'Y' medium (Table 1) to which increasing concentrations [0.2 up to 2.0% (w/v)] were added. In other experiments 0.06 M PBS instead of NaCl 85 g l⁻¹ was used in the 'Y' medium which otherwise was of the same composition as that described in Table 1. Finally the carbon sources in the 'Y' medium viz Bacteriological peptone (15 g l⁻¹) and

Casein hydrolysate (5 g l⁻¹) were in some tests replaced by either 20 g l⁻¹ of Bacteriological peptone or by 0.5% Casein hydrolysate.

Preparation of the 'Y' Medium

The medium constituents were suspended in distilled water holding approximately 10 °C which was then heated to boiling in order to achieve complete dissolution. After sterilisation viz autoclaving at 121 °C for 15 minutes the medium was immediately cooled to 45-50 °C and the pH adjusted to 7.4. The medium was dispensed into plastic dishes in an amount of 22.5 g per dish which were stored over blue gel in a plastic bag at room temperature until use.

Other Media Tested

Horse blood agar (HBA) consisting of Blood agar base no 2 (Oxoid batch no 22 611 ED 2 127) supplemented with 4% defibrinated horse blood; DC agar (Oxoid batch no 199984 DN 15 442); YS agar (see reference 19) (Merck batch no 6 393 193) and MacConkey agar (Difco batch no 646079) were prepared in accordance with the instructions of the suppliers and LSU according to those of Juhlin & Ericson (6).

Recovery Tests

In one series of experiments each of 224 faecal specimens were infected with one of altogether 40 strains of *Y. enterocolitica* belonging to serotypes 3 (25 strains), 6 (2 strains), 8 (2 strains) and 9 (11 strains). The infected stools were cultured on the DC, MC, LSU, YS and 'Y' media. The bacteria were suspended in isotonic phosphate buffered saline (PBS) pH 7.4 to a density of approximately 10⁴⁻⁵ cells ml⁻¹ as determined by viable count on HBA plates after incubation at 22 °C and 29 °C for two days. The faecal specimens used were selected among stools sent to the Bacteriological Department University Hospital Lund which were found not to contain *Salmonella* and *Shigella* spp. or *Y. enterocolitica*. Each of the faecal specimens tested was diluted 1:100 in 10 ml PBS pH 7.4. To these suspensions 0.1 ml of one of the above mentioned *Y. enterocolitica* preparations were admixed. Of the infected faecal specimens 0.1 ml were used to inoculate all media tested.

Recovery of *Y. enterocolitica* from Clinical Specimens

One hundred faecal specimens sent to the Bacteriological Laboratory University Hospital Lund during 1 month period for the diagnosis of a possible yersiniosis were studied for *Y. enterocolitica*. The specimens were inoculated on DC agar and incubated at 22 °C and 29 °C for 78 hours and in enrichment buffer consisting of NaCl 8.5 g, Na₂HPO₄ 8.4 g, KH₂PO₄ 1.1 g diluted in 1 l of demineralized water. The inoculated buffer was incubated at 4 °C for 3 weeks. Finally the specimens were cultured on the 'Y' medium which was incubated also for 2 days at 22 °C and 29 °C.

In the experimental system used in this study the *Y. enterocolitica* medium was found superior to the *Y. enterocolitica* and *Y. enterocolitica* media in terms of selectivity and differentiating properties for the recovery of *Y. enterocolitica* from faecal specimens. In experimentally infected

from this period is in principle similar to that reported from the study of the series of 100 specimens described above.

The high selectivity of the *Y. enterocolitica* medium favours the detection of *Y. enterocolitica* as compared to other media in current use for the isolation of the organism from faecal specimens. By the use of the *Y. enterocolitica* medium the time-consuming cold enrichment can be omitted without decreasing the recovery frequency. The medium is cheap and easy to prepare.

faecal specimens is dependent on the efficiency by which the indigenous intestinal flora, particularly *Proteus* and *Pseudomonas* spp. is suppressed. By the addition of sodium oxalate and bile salts to the *Y. enterocolitica* medium growth of gram positive cocci was completely suppressed. By these additives the medium was also made differentiating for *Y. enterocolitica* on one hand and other gram negative rods on the other. To increase the differentiating

distinct colour change of the medium

Room temperature has been indicated as the optimal incubation temperature for the isolation of *Y. enterocolitica*.

Although larger colonies of *Y. enterocolitica* were seen on the *Y. enterocolitica* medium when incubated at 37°C there was a pronounced

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did virtually not occur.

When testing pure cultures of *Y. enterocolitica* pin point size colonies of all strains tested emerged already 24 hours after incubation at 29°C when using HBA medium. Under similar culture conditions some such strains developed visible colonies first after 48 hours of incubation when using the *Y. enterocolitica* medium. Neither on LSU or on *Y. enterocolitica* medium were any colonies detectable after incubation at 37°C for 24 hours while on DC agar sometimes pin point colonies were found after that period of incubation. Thus in isolation studies on *Y. enterocolitica* we recommend that the *Y. enterocolitica* medium is incubated for at least 48 hours before discarded as negative.

The *Y. enterocolitica* medium has now been in use for approximately 7 years at the Bacteriological Laboratory, University Hospital, Lund. The experience

Table 4 Reisolation of *Yersinia enterocolitica* on the «Y» Medium Containing either Sodium Oxalate (OXL) 1-ketoglutarate (KGL) or Sodium Succinate (SCC) (all Substances at a Concentration of 0.06 M) and on Horse Blood Agar (HBA) from 40 Experimentally Infected Faecal Specimens. The Plates Were Incubated at 29 °C for 48 Hours before Read. The Inoculum Used Resulted in Approximately 30 Colony-forming Units per Plate. Number of Strains Growing and Diameter of Colonies

Medium	Number of strains growing (%)	Diameter of colonies (mm)	
		Mean	Range
OXL	40 (100)	2.3	1.3-2.5
KGL	32 (80)	1.3	1.0-1.6
SCC	36 (90)	1.6	1.3-1.8
HBA	40 (100)	2.0	1.3-2.3

(w/v) was found to give the highest reisolation frequency and also colonies with the largest diameter. However, 0.8-1.0% of sodium oxalate resulted in the highest selectivity (Table 5). In the set of experiments in which NaCl was replaced by PBS the acid produced from fermentation of lactose by gram negative bacteria caused a less distinct colour change of the indicator, viz. neutral red.

Comparison of the Recovery of *Y. enterocolitica* from Infected Stools Using Different Media

When the «Y» medium was incubated at 29 °C for 2 days, *Y. enterocolitica* could be reisolated from 99.5% of the 224 experimentally infected specimens, while when using DC, MC, LSU and YS, the bacterium could be recovered from only 90% or less of the specimens (Table 3).

Recovery of *Y. enterocolitica* from Clinical Specimens

Altogether 13 strains of *Y. enterocolitica* were isolated from the 100 clinical specimens of faeces studied. One strain did only grow on the «Y» medium, while the 12 other strains were recovered both on DC agar and after cold enrichment.

The largest colonies of *Y. enterocolitica* on the «Y» medium were found when the medium was incubated at 29 °C. Thus the average colony diameter after 48 hours of incubation at this temperature was 2.3 mm, as compared to 2.2 and 1.9 mm at 22 °C and 37 °C, respectively.

DISCUSSION

Y. enterocolitica grows on simple inorganic media provided the presence of certain growth promoting factors, e.g. thymidine. Some strains of this species may also grow on minimal media containing ammonium sulphate as nitrogen source and glucose as the only source of carbon and energy (2).

Media commonly used for the isolation of *Y. enterocolitica* from clinical specimens consist of peptones, or meat infusions supplemented with lactose and different agents promoting selectivity, such as sodium desoxycholate, potassium tetrathionate, bile salts, brilliant green and crystal violet (6, 17, 19).

In cultures of faecal specimens, cold temperature enrichment has been used to overcome the problem of overgrowing contaminants (4, 5, 12, 18, 19). Thus inoculation of such specimens into Rappaport broth (4, 19) or phosphate buffered saline (5, 12, 18), followed by incubation at 4 °C for 3 weeks, favours the recovery of *Y. enterocolitica* from stools (12).

Table 5 Recovery of *Yersinia enterocolitica* from Experimentally Infected Faecal Specimens after 48 Hours Incubation at 29 °C on the «Y» Medium Containing Increasing Concentrations of Sodium Oxalate. Diameter of Colonies and Grading of Selectivity

Concentration (g (w/v)) of sodium oxalate used	No. of faecal specimens yielding growth (%)	Diameter of colonies (mm)		Degree of selectivity ^a
		Mean	Range	
0.2	26 (65)	PP ^b	PP	3
0.4	29 (73)	PP	PP	3
0.6	35 (88)	1.7	1.3-1.8	2
0.8	38 (95)	2.2	1.2-2.6	1
1.0	40 (100)	2.1	1.2-2.5	1
2.0	8 (20)	PP	PP	3

^a 1 - high, 2 - intermediate, 3 - low

^b PP - pin point colonies

SEROLOGY OF *NEISSERIA GONORRHOEAE* CHARACTERIZATION OF RABBIT HYPERIMMUNE ANTISERA BY LINE-ROCKET IMMUNOELECTROPHORESIS FOR USE IN CO- AGGLUTINATION

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Sandström E & Danielsson D Serology of *Neisseria gonorrhoeae* Characterization of rabbit hyperimmune antisera by line rocket immunoelectrophoresis for the use in co-agglutination Acta path microbiol scand Sect B 88 17-26 1980

The co-agglutination (COA) and line rocket immunoelectrophoresis (L RIE) techniques were investigated for the serological classification of *Neisseria gonorrhoeae* COA with absorbed sera was found to demonstrate strain specificity as well as cross reacting antigens The results were dependent on the

findings by the L RIE tests selective absorptions of antibodies were performed for use in preparation of COA reagents It was found that the reactions of these reagents with the MOVIP reference strains could be assigned to three different antigenic classes Reagents prepared for these classes are proposed as a basis for serological classification of *Neisseria gonorrhoeae*

Key word *Neisseria gonorrhoeae* co-agglutination immunoelectrophoresis serology classification

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Serological classifications of the various bacterial genera and species such as *Salmonellae*, *Shigellae*, *Escherichia coli* and *Neisseria meningitidis* have greatly aided the understanding of the epidemiology and pathogenesis of infections with these organisms as well as their ecology Such classification has not yet been worked out and accepted for *Neisseria gonorrhoeae* despite serious attempts by several investigators In these studies viable or killed whole gonococcal cells were used in standard agglutination (17-19) complement fixation (14) & direct or indirect immunofluorescence (IFL) (6

13-18) Other investigators used gonococcal cell fractions in various stages of purification in agar gel diffusion (6-9-10) passive haemagglutination or haemolysis (1-4-12) All the published data demonstrated the serological heterogeneity of these organisms

We recently reported the results of co-agglutination (COA) and indirect IFL in demonstrating strain specific antigens of *N. gonorrhoeae* by the use of cross absorbed antibodies (8) The results correlated well with those obtained by rocket line immunoelectrophoresis with antigen-containing intermediate agar gel Our findings also indicated that

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Performance of COA tests One drop of a 1% suspension (w/v) of reagent staphylococci was mixed on glass slide with one drop of the 1% gonococcal suspension. The slide was then tilted approximately once very second for 2 min after which the reaction was read in oblique light. Negative reactions were recorded as - and positive as 1+ (weak) 2+ (moderate) and 3+ strong and very strong)

Line rocket immunoelectrophoresis (L. RIE)

The lysates of sonicated gonococcal cells (250 mg ml⁻¹ related to the wet weight) were used in comparative studies in the L. RIE tests with homologous and heterologous antigen antibody combinations arranged as shown in Fig. 1.

The buffer in the electrophoresis vessels ionic strength 0.1 pH 8.6 was recirculated and the electrophoresis apparatus was cooled with tap water during the test runs.

The area of the glass plate covered by antibody gel was about 75 cm² and the reference antigen line gel at the cathodic end of the plate about 10 cm². Each rectangular agarose area with antigen of the intermediate gel measured about 0.25 cm² and was separated by a corresponding rectangular agarose gel without antigen. The rectangular gels were made in a mould composed of two glass plates separated by a U shaped frame 45 x 100 mm 2 mm thick. 0.3 ml agarose was carefully placed at the bottom (45 mm wide) of the mould. After solidification 0.3 ml anti gonococcal antiserum was added and this mixture of 2 parts against 1 part antigen was poured in after which the rest of the glass plate 75 x 100 mm was covered with a mixture of 13 ml agarose and 0.3 ml (in some tests 0.6 ml) anti gonococcal antiserum.

Electrophoresis was run for 22 h with a gradient of 15 V cm. The plates were then pressed between filter papers, washed twice in saline and once in water, pressed again and stained with Coomassie blue as described by Arlesen et al. (13).

1) Detection of «Strain Specific» Antigens and Cross Reacting Antigens among Gonococcal Strains by Co-agglutination Influence of Treatment of Antigens

We have showed previously that COA was a suitable method for demonstrating both «strain specific» and cross reacting antigens among gonococcal strains (8). These studies were extended in the present work with antigenococcal antibodies from ten rabbits immunized with the five S strains 14984 15057, 15000 11376 and 14190 (two rabbits for each strain). Reagent staphylococci were coated with antibodies unabsorbed or cross absorbed with the five strains. Results of COA tests performed with untreated formalin fixed or boiled whole gonococcal cells are shown in Table 1.

All five strains cross reacted with the unabsorbed anti GC antibodies obtained from the ten rabbits. These reactions were due in part to common antigens. Irrespective of the absorbing strain cross absorbed antibodies contained reactivity against heat stable antigens of the homologous strains. These antigens could therefore be considered as «strain specific» antigens. Cross absorbed antibodies also showed reactivity with some of the formalin fixed

ones somewhat more reactive and boiled whole cells the most reactive. The antigens responsible for these reactions could therefore be considered as cross reactive antigens which like the «strain specific» antigens seem to be heat stable.

Strains that appeared to be closely related by absorption and agglutination may appear unrelated when used as immunogens. This is exemplified by the anti 15000 antiserum which after absorption with strain 14190 reacted only with strain 15000. Furthermore strain 14190 also gave more reactions with the other absorbed anti 15000 antisera than any of the other absorbing strains. On the other hand strain 15000 did not react with any of the absorbed antisera against strain 14190.

2) Demonstration of Cross Reactive Antigens with Line rocket immunoelectrophoresis

COA tests showed that in addition to «strain specific» antigens most anti GC antisera contained

besides »specific« antigens, there were antigens showing cross reactivity between the strains which was dependent on which particular strain was used for absorption of the anti gonococcal antibodies.

In the present work the COA technique was used for further study of the serology of *N. gonorrhoeae*. Line rocket immunoelectrophoresis was developed to demonstrate the relationship between 16 selected gonococcal strains which could be grouped with regard to cross reacting antigens. With this technique information was rapidly obtained regarding the choice of absorbing strains to be used for the preparation of defined antibodies in COA.

MATERIALS AND METHODS

Neisseria gonorrhoeae Strains

A total of 21 strains were used in the present study. Five strains S 14984 S 15057 S 15000 S 11376 and S 14190 were isolated in Stockholm in 1973 from patients with uncomplicated gonorrhoea. These were selected on the basis of the results obtained from investigation of the immune response in complicated and uncomplicated gonorrhoea using the gonococcal complement fixation test (15). The 16 reference strains for the major outer membrane protein (MOMP) serotyping system (10) were kindly provided by Dr A. H. Johnston Houston Texas designated A 1 B 2 C 3 D 4 E 5 F 6 G 7 H 8 9 N 10 R 11 S 12 T 13 U 14 V 15 and W 16. The strains were cultured on the colony morphology typing medium (CMT) described by Aellegge *et al* (11) for 22 h in 5% CO₂ at 37 °C unless otherwise stated. The MOMP reference strains were received unselected as regards colony morphology type. T2 colonies with golden brown or dark brown colour were obtained from strains D 4 F 6 H 8 R 11 V 15 and T 13 while T1 like colonies of varying colour (light golden brown) had to be accepted for A 1 C 3 E 5 N 10 S 12 and U 14. T3 like for B 2 and G 7 and T5 like for 9 (1). They were selected and recultured daily over a two week period before freezing at -70 °C in narrow tubes from which not all samples could be thawed when needed (8). Cells of strains with dark brown colonies of the various types were preferred for use in the subsequent experiments.

Production of Antisera

Preparation of whole cell antigens. Organisms of T2 colony morphology types of the five S strains were cultured on CMT medium and seeded on agar plates

7.4 and treated with 2.5% formalin as described previously (5). The organisms were suspended in saline to a concentration corresponding to McFarland No. 4 and used for immunization within two weeks.

Preparation of sonicated antigens. Organisms of the 16

gonococcal MOMP serotypes A 1 to W 16 (10) were cultured on CMT medium in the same way as the colony morphology types described above. The organisms were harvested in PBS pH 7.4 centrifuged for 15 min at $2500 \times g$ and then adjusted to a concentration of 20 mg ml⁻¹ in PBS. The cells were disrupted by sonication with a 100 watt MSE ultrasonic apparatus as described previously (8). Sonicated cells prepared in this way were used for absorption of antiserum as described below. For immunization of rabbits the sediment was obtained by centrifuging the lysate at $30000 \times g$ (Beckman 55 ultracentrifuge) for 20 min washing once in saline and finally suspending in saline to 25 times the original volume of the lysate. The sediment was stored in small aliquots at -70 °C until use.

Immunization. White female rabbits weighing approximately 2.5 kg were used. Two rabbits were immunized with the formalin fixed whole cells for each of the five strains with an initial intramuscular injection of 2 ml antigen mixed with 2 ml Freund's complete adjuvant. Two weeks later the animals were given a series of 11 injections viz 0.1 ml 0.25 ml 0.5 ml and 1.0 ml of the same antigen at intervals of 3-4 days. The series was repeated after two weeks and the animals were bled two weeks after the last injection. Animals immunized with the sediment of the 16 gonococcal MOMP serotypes of Johnston *et al* (see above) were given a total of 11 subcutaneous injections at intervals of two weeks each time with 2 ml antigen mixed with 2 ml Freund's complete adjuvant. The animals were bled two weeks after the fourth injection.

Absorption of Antiserum

Absorption was performed by mixing one volume of antiserum with four volumes of the sonicated cells (see above) and incubating for 1 h in water bath at 37 °C and then overnight at +4 °C. The absorbed antiserum which in this way was diluted 1:5 was recovered by centrifugation at $30000 \times g$ for 20 min and if not used the same day stored at -70 °C until use. On some occasions undiluted antisera were absorbed with the corresponding volume of packed formalin fixed cells for 4 h at 37 °C and overnight at +4 °C. The antiserum was recovered by centrifugation and if residual activity to the absorbing strains persisted the absorption was repeated two or three times with heat treated cells for the same time and with the same concentrations.

C agglutination (COA)

Antigens. The gonococcal strains were grown on CMT medium. The cells were harvested in PBS pH 7.4 and used as untreated whole cells (UWC) with the addition of 0.1% Na₂S₂O₅ (w/v) as formalin fixed whole cells (FWC) (5) or as heated whole cells (HWC) after boiling for 1 h in a water bath. The gonococcal suspensions were adjusted to a 1% concentration (w/v).

Preparation of reagents. The production of protein A staphylococci reagent staphylococci sensitized with unabsorbed or absorbed antigenogonococcal antibodies and control reagents followed the procedure described previously (8).

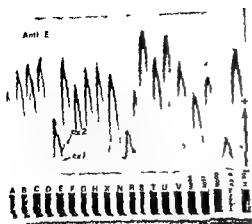


Fig 1 Line rocket immunoelectrophoresis with anti E 5 in the reference antibody gel sonicated cells of strain E 5 in the line gel and sonicated cells of homologous and heterologous strains in the intermediate gel

cx 1 and cx 2 designate two different precipitin lines parallel to the line gel connecting rockets formed over the antigens in the intermediate gel (type 1)

Capital letters refer to the MOMP reference strains

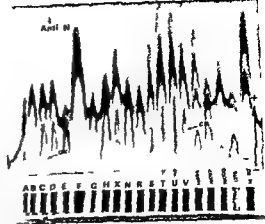


Fig 3 Line rocket immunoelectrophoresis with anti N 10 in the reference antibody gel sonicated cells of strain C 3 in the line gel and sonicated cells of homologous and heterologous strains in the intermediate gel

cx designates precipitin line parallel to the line gel connecting rockets over antigen in the intermediate gel (type 1 reaction)

cr designates rocket over antigen in the intermediate gel not connected to a visible line parallel to the line gel (type 2 reaction)

Capital letters refer to the MOMP reference strains

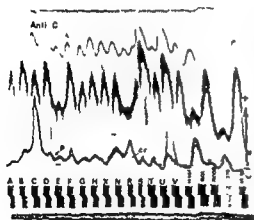


Fig 2 Line rocket immunoelectrophoresis with anti C 3 in the reference antibody gel sonicated cells of strain C 3 in the line gel and sonicated cells of homologous and heterologous strains in the intermediate gel

pr designates partial reaction of identity between the precipitin line parallel to the line gel and the rocket (type 1 reaction)

cr designates rocket over antigen in intermediate gel not connected to a visible line parallel to the line gel (type 2 reaction)

Capital letters refer to the MOMP reference strains

were at times seen as partial reactions (pr Fig 2) In the type 2 reaction a rocket line formed by the antigens of the intermediate gel could be identified but no line of identity connected these rockets with the concentrations of antiserum used and antigen in the line gel (cr Fig 2) These precipitin lines represented another antigen or antigens related to the homologous strain In the type 3 reaction inverted or no rockets formed over the antigens in the intermediate antigen gel These reactions (designated \rightarrow) indicated lack of specificity or cross reacting antigens In the type 4 reaction antigens in the intermediate agar gel formed rocket precipitin lines with reactions of identity at the anodic end of the agarose (Fig 1 and 2) for all the 16 MOMP strains These rockets represented the common antigens Examples of two independent type 1 reactions are shown in Fig 1 one with strains B 2 D 4 E 5 G 7 R 11 and V 15 (cx1) and the second with strains B 2 E 5 G 7 H 8 N 10 and V 15 (cx2) in the test with the E 5 anti E 5 combination The C-3 anti C-3 combination (Fig 2) illustrates type 1 reaction for strains B 2 C-3 E 5 F 6 G 7 H 8 N 10 and R 11 Partial identity can be seen in Fig 2 for strains D 5 and V 15 Type 2 reactions were obtained with strains C 3 D-4 R 11 and V 15

TABLE I Co-agglutinations of GC Cells with Reagent Staphylococci Coated with Anti-GC Antibodies Absorbed x Homologous or Heterologous N gonorrhoeae Organisms Reactions Graded as Described in Material and Methods

Antiserum against strain (rabbit) ^a	Absorbing strain	COA reactions with untreated formalin treated and heated whole cells of <i>Neisseria gonorrhoeae</i>											
		S 14984			S 15057			S-15000			S 11376		
		UWC ^b	FWC ^c	HWC ^d	UWC	FWC	HWC	UWC	FWC	HWC	UWC	FWC	HWC
S 14984	-	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(b ₁ /b ₂)	S 15000	3+	3+	3+	-/2+	-/2+	2+	-	-	2+/-	-/2+	+/3+	3+
	S 15057	3+	3+	3+	-	-	2+/-	-	-	-	-	3+/-	2+/-
	S 11376	2+	3+	3+	-	-	2+/-	-	+	2+/-	-	-	2+/-
	S 14190	2+	2+	2+	-	-	-	-	-	-	-	+	-
	■ 14984	-	-	-	-	-	-	-	-	-	-	-	-
S 15057	-	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(d ₁ /d ₂)	S 14190	+	2+/-	3+/-	3+	3+	3+	-	2+/-	3+/-	+	3+/-	3+/-
	■ 15000	-	-	3+/-	3+	3+	3+	-	-	+	-	3+/-	+
	S 14984	-	-	3+/-	3+	3+	3+	-	+	3+/-	-	+	3+/-
	S 11376	+	-	-	3+	3+	3+	-	-	2+/-	-	-	-
	S 15057	-	-	-	-	-	-	-	-	-	-	-	-
S 15000	-	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(f ₁ /f ₂)	S 14984	-	-	-	-/2+	-/2+	2+	3+	3+	3+	-	-/2+	-/2+
	S 15057	-	-	+	-	-	-	3+	3+	3+	-	-/3+	+
	S 11376	-	-	-	-	-	-/2+	3+	3+	+/3+	-	-	-
	S 14190	-	-	-	-	-	-	3+	3+	+/3+	-	-	+
	S 15000	-	-	-	-	-	-	-	-	-	-	-	-
S 11376	-	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(c ₁ /c ₂)	S 14190	+	+	2+	-/2+	2+	+	-	-	-	3+	2+	3+
	S 15000	+	+	3+	+	+	+	-	-	-	2+	2+	3+
	S 15057	+	+	3+	-	-	-	-	-	-	2+	2+	3+/-
	S 14984	-	-	-	+	-	-	-	-	-	2+	2+	2+
	S 11376	-	-	-	-	-	-	-	-	-	-	-	-
S 14190	-	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
(e ₁ /e ₂)	S 15057	-/2+	2+	3+	-	-	-	-	-	+	-	-	2+/-
	S 15000	-/2+	+	3+	-	-	-	-	-	-	-	-	2+/-
	S 14984	-	-	-	-	-	-	-	-	-	-	-	2+
	S 11376	-	-	+	-	-	-	-	-	-	-	-	2+/-
	S 14190	-	-	-	-	-	-	-	-	-	-	-	-

^a COA reactions of the individual rabbit presented only when there was a difference of more than 1 +

^b UWC - untreated whole cells ^c FWC = formalin treated whole cells ^d HWC - heated (100 °C) whole cells

antibodies to more than one cross-reactive antigen, thus resulting in complex patterns. To cover all combinations with 10 strains and their corresponding antisera, 100 absorptions and 1000 tests would be required if the relationship was unknown. However, we have shown in a previous work that the results of COA tests coincided well with those obtained by rocket line immunoelectrophoresis (8). In the present work, we therefore developed the line-rocket immunoelectrophoresis described in Materials and Methods, with the aim of guiding and reducing the number of absorptions for anti GC antibodies for use in COA tests. We decided to use hyperimmune rabbit antisera against the 16 gonococcal serotypes described by Johnston *et al* (10) known to differ in at least one antigen. Antisera, one for each strain, that gave a precipitation pattern

typical of previously described strain-specific antigens (8), were selected for these studies.

In these L-RIE tests, antigen preparations from more than 20 GC strains could be compared with a reference antigen-antibody system and four types of reactions could be observed. These are referred to as type 1 type 2 type 3 and type 4 reactions. In the type 1 reaction, a line of identity connected the precipitin line of the reference antigen line gel with the rocket(s) formed by the antigens of the homologous and one or more of the heterologous strains in the intermediate gel. These precipitin lines thus represent cross-reacting (cx, Fig. 1) antigen complexes between the homologous and the heterologous strains. One or two such cross-reacting antigen-antibody systems could be detected in a particular test run. Dissociations of the

TABLE 3 Co agglutination Reactions of Heated (100 °C 1 h) whole Gonococcal Cells from the MOMP Reference Strains with Reagent *Staphylococci* Coated with Anti GC Antibodies Absorbed with Homologous and Heterologous Strains Reactions Graded as Described in Material and Methods

Serum inst ant abs (t)	Absorbing strain	Results of COA tests with MOMP reference strains																	
		W	B	G	E	R	D	V	N	H	C	X	S	T	U	A	F		
5 S 32)	B 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	G 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	E 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	R 11	-	2+	3+	3+	-	-	+	+	+	-	-	-	-	-	-	-		
	D 4	-	2+	2+	+	+	-	-	-	+	-	-	-	-	-	-	-		
	V 15	-	2+	3+	3+	+	+	+	+	-	-	-	-	-	-	-	-		
	N 10	-	3+	3+	3+	3+	+	2+	-	-	-	-	-	-	-	-	-		
	H 8	-	3+	3+	3+	3+	+	2+	-	-	-	-	-	-	-	-	-		
A 1	-	3+	2+	3+	3+	+	2+	3+	3+	-	-	-	-	-	-	-			
4 S 31)	W 16	-	3+	2+	3+	3+	2+	3+	2+	2+	3+	-	-	-	-	+	2+		
	B 2	-	-	-	-	3+	3+	3+	-	-	3+	-	-	-	-	-	-		
	G 7	+	+	-	2+	2+	3+	3+	-	-	3+	-	-	-	-	-	-		
	E 5	-	-	-	-	+	3+	2+	-	-	-	-	-	-	-	-	-		
	R 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	D 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	V 15	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-		
	C 3	2+	+	+	+	3+	3+	3+	-	-	-	-	-	-	-	-	-		
	A 1	-	2+	-	-	3+	3+	3+	-	-	2+	-	-	-	-	-	-		
	F 6	nd*	3+	2+	3+	3+	3+	3+	+	+	3+	-	-	-	1+	-	-		
C 3+B 2	-	-	-	-	-	3+	3+	-	-	-	-	-	-	-	-	-			
10 S 37)	B 2	nd	-	-	-	+	3+	-	3+	3+	3+	3+	3+	-	-	2+	2+		
	G 7	nd	-	-	2+	+	3+	+	3+	3+	3+	3+	2+	-	-	2+	2+		
	R 11	-	-	-	+	-	3+	-	3+	2+	+	3+	-	-	-	2+	-		
	D 4	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	-	-	-	-		
	V 15	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	-	+	-	-		
	N 10	-	-	-	-	+	-	-	3+	-	-	-	-	-	-	-	-		
	H 8	-	-	-	-	+	-	-	3+	-	-	-	-	-	-	-	-		
	C 3	-	-	-	+	-	+	-	2+	+	-	2+	-	-	-	-	-		
	V 9	-	+	+	+	2+	2+	-	2+	2+	-	-	-	-	-	+	-		
	S 12	-	+	-	2+	-	+	+	3+	3+	-	3+	-	-	-	2+	-		
	U 14	-	-	-	2+	-	3+	-	3+	2+	+	2+	-	-	-	2+	-		
12 S 39)	W 16	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	3+	3+	2+	-		
	B 2	2+	-	-	-	2+	3+	-	3+	3+	3+	3+	3+	3+	3+	3+	-		
	R 11	-	-	-	-	-	-	-	3+	2+	2+	3+	3+	3+	3+	-	-		
	N 10	-	-	-	-	-	-	-	-	-	-	-	2+	-	-	-	-		
	H 8	2+	-	-	-	-	-	-	-	-	-	-	3+	-	-	-	-		
	C 3	2+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	V 9	-	-	-	-	-	-	-	-	+	-	+	2+	-	2+	-	-		
	S 12	-	-	-	-	-	-	-	-	+	-	-	3+	-	-	-	-		
	T 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-		
	U 14	-	-	-	-	-	-	-	-	+	-	-	2+	-	-	-	-		
	A 1	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	3+	3+	-	-		
* nd not done																			

* nd not done

TABLE 2 *Results with Line-rocket Immunelectrophoresis Using Antisera to the MOMP Reference Strains T_{Es} against Sonicated Cells of the Homologous and Heterologous Strains in the Intermediate Gel*

Antiserum against strain (rabbit)	Antigen in line gel	L-RIE reactions with antigens from the MOMP reference strains in the intermediate															
		W	B	G	E	R	D	V	N	H	C	X	S	T	U	A	
B-2 (KS 29)	B-2	nd —	cx ^b cr ^d	cx cr	cx cr	cx —	cx —	pr ^c —	cx —	cx —	— —	— —	— —	— —	— —	— —	
G-7 (KS 34)	G-7	— cr	cx —	cx cr	cx —	cx —	— —	— —	cx —	cx —	— —	— —	— —	— —	— —	— —	
E-5 (KS 32)	E-5	— — —	cx ¹ cx ² —	cx ¹ cx ² —	cx ¹ cx ² cr	cx ¹ — cr	cx ¹ — —	cx ¹ cx ² —	— cx ² —	— cx ² —	— — —	— — —	— — —	— — —	— — —	— — —	
R-11 (KS 38)	R-11	nd ^e nd	cx ¹ —	cx ¹ —	cx ¹ —	cx ¹ cx ²	cx ¹ cx ²	cx ¹ cx ²	cx ¹ —	cx ¹ —	cx ¹ cx ²	— —	— —	— —	— —	— —	
D 4 (KS 31)	D 4	nd nd	— cr	— cr	— cr	— cr	cx cr	cx cr	— —	— —	— cr	— —	— —	— —	— —	— —	
V-15 (KS 42)	V-15	nd nd	— cr	cx cr	cx cr	cx —	cx cr	cx cr	cx —	cx —	cx cr	— —	— —	— —	— —	— —	
N-10 (KS 37)	N-10	nd nd	— —	— —	— cr	— —	— —	— —	cx cr	cx cr	cx —	cx —	cx —	— —	— —	— —	
H-8 (KS 44)	H 8	— —	cx —	cx —	cx —	— —	— —	— —	cx —	cx —	— cr	— —	— —	— —	— —	— —	
C-3 (KS 30)	C-3	— —	cx —	cx —	cx —	cx cr	pr cr	pr cr	cx —	cx —	cx cr	— —	— —	— —	— —	— —	
X-9 (KS 36)	X-9	— — —	— cx —	— cx —	— cx cr	— — cr	— — —	— — —	— — cr	— — —	— cx cr	cx —	— — —	cx cr	cx cr	— — —	
S-12 (KS 39)	S 12	— — —	— — —	— — —	— — —	— — —	— — —	— — —	cx ¹ cx ² cr	cx ¹ cx ² —	— cx ² —	— — cr	cx ¹ cx ² —	— cr	— cr	— cr	
T-13 (KS 40)	T-13	— — —	— — —	— — —	— — —	— — —	— — —	— — —	— cx ² cx ³	— — —	— cx ² —	— — —	— — —	cx ¹ cx ² cx ³	cx ¹ cx ² cx ³	— — —	
U-14 (KS 41)	U 14	nd nd	— —	— —	cx —	— —	— —	cx —	cx —	cx —	cx —	— —	— —	cx cr	cx cr	— —	
A-1 (KS 28)	A 1	— —	— —	— —	— —	— —	— —	— —	— —	cx —	— —	— cr	— —	— —	— —	cx cx	
F-6 (KS 43)	F 6	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —	— —	

- a — = type 3 reaction: no rocket formed over the antigen in the intermediate gel
 b cx = type 1 reaction: see text and Fig. 1
 c pr = partial reaction (cf Fig. 2)
 d cr = type 2 reaction (cf Fig. 2)

TABLE 3 Co agglutination Reactions of Heated (100 °C 1 h) whole Gonococcal Cells from the MOMP Reference Strains with Reagent Staphylococci Coated with Anti GC Antibodies Absorbed with Homologous and Heterologous Strains Reactions Graded as Described in Material and Methods

titrum antibody (b1)		Absorbing strain	Results of COA tests with MOMP reference strains															
			W	B	G	E	R	D	V	N	H	C	X	S	T	U	A	F
5 S 32)	B 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	G 7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	E 5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	R 11	-	2+	3+	3+	-	-	+	+	+	+	-	-	-	-	-	-	-
	D 4	-	2+	2+	+	+	-	-	-	+	+	-	-	-	-	-	-	-
	V 15	-	2+	3+	3+	+	+	-	-	+	-	-	-	-	-	-	-	-
	N 10	-	3+	3+	3+	3+	+	2+	-	-	-	-	-	-	-	-	-	-
	H 8	-	3+	3+	3+	3+	+	2+	-	-	-	-	-	-	-	-	-	-
A 1	-	3+	2+	3+	3+	+	2+	3+	3+	-	-	-	-	-	-	-	-	
4 S 31)	W 16	-	3+	2+	3+	3+	2+	3+	2+	2+	2+	3+	-	-	-	-	+	2+
	B 2	-	-	-	-	3+	3+	3+	-	-	-	3+	-	-	-	-	-	-
	G 7	+	+	-	2+	2+	3+	3+	-	-	-	3+	-	-	-	-	-	-
	E 5	-	-	-	-	+	3+	2+	-	-	-	-	-	-	-	-	-	-
	R 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	D 4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	V 15	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-
	C 3	2+	+	+	+	3+	3+	3+	-	-	-	-	-	-	-	-	-	-
	A 1	-	2+	-	-	3+	3+	3+	-	-	-	2+	-	-	-	-	-	-
	F 6	nd*	3+	2+	3+	3+	3+	3+	+	+	3+	-	-	-	-	2+	-	-
	C 3 + B 2	-	-	-	-	-	3+	3+	-	-	-	-	-	-	-	-	-	-
10 S 37)	B 2	nd	-	-	-	+	3+	-	3+	3+	3+	3+	3+	3+	-	-	2+	2+
	G 7	nd	-	-	2+	+	3+	+	3+	3+	3+	3+	3+	2+	-	-	2+	2+
	R 11	-	-	-	+	-	3+	-	3+	2+	+	3+	-	-	-	-	2+	-
	D 4	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	3+	-	-	-	-
	V 15	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	3+	-	+	-	-
	N 10	-	-	-	-	-	-	-	-	-	-	3+	3+	3+	-	-	-	-
	H 8	-	-	-	-	+	-	-	3+	-	-	-	-	-	-	-	-	-
	C 3	-	-	-	+	-	+	-	2+	+	-	-	-	-	-	-	-	-
	X 9	-	+	+	+	2+	2+	-	2+	2+	-	-	-	-	-	-	+	+
	S 12	-	+	-	2+	-	+	+	3+	3+	-	3+	-	-	-	-	2+	-
	U 14	-	-	-	2+	-	3+	-	3+	2+	+	2+	-	-	-	-	2+	-
12 KS 39)	W 16	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	3+	3+	3+	2+	-
	B 2	2+	-	-	+	2+	3+	-	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
	R 11	-	-	-	-	-	-	-	3+	2+	2+	3+	3+	3+	3+	3+	-	-
	N 10	-	-	-	-	-	-	-	-	-	-	-	-	2+	-	-	-	-
	H 8	2+	-	-	-	-	-	-	-	-	-	-	-	3+	-	-	-	-
	C 3	2+	-	-	-	-	-	-	-	+	-	+	2+	-	2+	-	-	-
	X 9	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	S 12	-	-	-	-	-	-	-	-	+	-	-	3+	-	-	-	-	-
	T 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	L 14	-	-	-	-	-	-	-	-	+	-	-	2+	-	-	-	-	-
	A 1	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	3+	3+	3+	-	-

*nd not done

On the basis of these findings, the strains could be arranged according to *type 1*, *type 2* and *type 3* reactions (Table 2). The strains could be placed in an apparently continuous pattern due to covarying cross-reacting antigens revealed by *type 1* reactions. In this way the MOMP strains could be divided into two main groups. The first consisted of strains B-2, G-7, E-5, R-11, D 4 and V-15, which with their corresponding antibodies formed one or two *type 1* reactions with three to six of these strains but not with strains S-12, T-13, U-14 or A-1. These strains were referred to the second group, together with strains N-10, H-8, C-3 and X-9 which gave *type 1* reactions in various combinations within both groups. Strains S-12, T-13, A-1 and F-6 gave only *type 1* reactions within the second group. *Type 2* reactions behaved similarly. By using heterologous antigens in the reference line gel (strain C-3 of the second group) (Fig. 3), *type 1* reactions were confined to the second group and *type 2* reactions in various combinations within the first and second groups.

3) Correlation between L-RIE and COA

Each antiserum used in L-RIE tests (Table 2) was absorbed separately with three different strains: one heterologous strain within the same group, one cross-reacting and one non-reacting strain of the other group. Reagent staphylococci coated with these antibodies were then used to test heated (100 °C) whole cells of the 16 MOMP reference strains. The reaction patterns obtained could be referred to three classes. The first consisted of absorbed antibodies against antigens common to strains within the same group (see L-RIE results in Table 2) as the immunizing strain, the second of absorbed antibodies against the immunizing strain (sometimes also against another closely related strain within the same group) and the third of absorbed antibodies against cross-reacting antigens of strains belonging to both groups. These results are shown in Table 3 with four selected antisera that were absorbed with the strains which gave *type 1* and/or *type 2* reactions and with a few which gave *type 3* reaction in the L-RIE.

Anti-E-5 Absorption of this antiserum with strains B-2, G-7 and E-5 showed that these strains shared most antigens corresponding to the *type 1* reactions with anti-E-5 in L-RIE (Table 2). Cross-reactive antigens were found in strains R-11, D-4 and V-15 as well as N-10 and H-8 when anti-E-5 was absorbed with a strain belonging to the second group in the L-RIE tests again indicating two covarying cross-reacting antigens: one belonging to class 1, the other to class 3.

Anti-D-4 The COA tests with absorbed anti-D-4

showed that strains R-11, D-4 and V-15 shared least two antigens. This could be shown by absorbing with strain B-2, after which reactions were obtained with R-11, D-4, V-15 of the same group and C-3 of the other group (class 3). After absorption with C-3, reactions were obtained with W-16, B-2, G-7, E-5, R-11, D-4 and V-15 (class 1). Only strains D-4 and V-15 reacted in the COA tests after absorption with B-2 and C-3 (class 2) which corresponds to the *type 1* reaction in the L-RIE. Reactions corresponding to classes 1 and 3 appeared as *type 2* reactions in L-RIE. It was also clear that the results of the COA tests were dependent on the strain used for absorption.

Anti-N-10 After absorption of this antiserum with strains D-4 or V-15, strong reactions were obtained with strains N-10, H-8, C-3, X-9 and S-12, which indicated that these strains shared the same antigens (class 1). This corresponded to *type 1* reactions in L-RIE. An almost monospecific antiserum to strain N-10 was obtained by absorption with H-8, which was closely related to N-10 according to L-RIE tests. After absorption with unrelated strains (for example U-14) numerous and often weak cross-reactions were observed.

Anti-S-12 A strain specific antigen of S-12 (class 2) was demonstrated after absorption with N-10 or U-14. The strong reactions with strains N-10, H-8, C-3, X-9, S-12, T-13 and U-14 after absorption with R-11 or A-2 correspond to antigens of the same group and can therefore be referred to class 1.

DISCUSSION

Most attempts to achieve serological classification of *Neisseria gonorrhoeae* have suffered from the assumption that a single major antigen exists in all gonococci which can vary only to a limited extent. Notable exceptions are the investigations by Wilson who from agglutination studies of whole organisms defined the antigenic pattern of a particular strain by a formula (19) and those by Maeland (12) who from studies of antigenic endotoxin fractions described the occurrence of varying numbers of antigenic factors in a particular strain. Usiné isolated outer membranes for serotyping gonococci by the Ouchterlony method. Johnston *et al.* (10) pointed out that even these purified antigens gave unwanted and disturbing antibodies if the immunization was not carefully supervised.

In the present work we showed by both coagglutination and immunoelectrophoresis techniques that rabbit hyperimmune anti-gonococcal antisera contained reactivity against common as

ell as strain-specific and cross reactive antigens these findings are in agreement with those of a previous report (8) and they further support the observations by others that the antigenic pattern of a particular gonococcal strain could best be described by a formula (12-19)

The COA method which is not disturbed by the inherent auto agglutinating properties of many GC strains was found suitable for detecting various antigens. However the total antigenic pattern of a particular strain was not revealed until the antibodies were extensively cross absorbed and cross tested with homologous and heterologous strains. Procedures that are laborious and time consuming (the results in Table 1) also show that the patterns obtained were complex and dependent on the immunizing, absorbing and agglutinating properties of a particular strain. They were also to a minor extent dependent on which individual rabbit immune serum was used. It was noteworthy that whole cells heated at 100 °C were more reactive than those untreated or formalin fixed. We have shown previously that for example untreated cells from 14 colony morphology types might even be unagglutinable though not after heat treatment (8). Unfixed organisms that do not react in a COA test should therefore also be tested after such treatment.

We showed previously that there was excellent agreement between results obtained with the COA method with cross absorbed antibodies and crossed and rocket line immunoelectrophoresis with antigen concentrations as represented by

when using a homologous antigen antibody system permitted comparison of the antigenic relationship between more than 20 strains. This is the advantage of L.R.I.E. as compared to rocket line and crossed immunoelectrophoresis.

The L.R.I.E. was applied to the 16 MOMP reference strains (10) which then could be divided into two main groups. Strains B 2 G 7 E 5 R 11 D-4 and V 15 were clearly separated from strains S 12 T 13 C 14 and A 1. Strains V 10 H 8 C 3 and V 9 reacted with antisera against both of these sets of strains and their corresponding antisera also reacted with both sets. The observed reaction patterns with strains B 2 G 7 E 5 V 10 H 8 and with strains R 11 D-4 V 15 C 3 led to the

and V 15 belonging to the first antigenic group. The second antigenic group of class 1 was represented by the rest of the MOMP strains. Class 3 consisted of strains in both these

groups. This concept was confirmed when absorptions of antisera for the COA test were carried out with three different strains i.e. absorption with a strain within the same antigenic group, absorption with a cross reacting strain and absorption with a non reacting strain respectively from the other antigenic group. Absorbed antibodies could then be produced with a reaction pattern in COA tests distinctive for class 1.

It was also found that closely related strains often absorbed all activity only occasionally leaving specific antisera against 1 or 2 MOMP reference strains. Antigens responsible for these COA reactions could then be referred to a third class. Antibodies absorbed with unrelated strains were often not suitable for analysis in COA tests because of multiple weak and complex reaction patterns thus contrasting with the strong reactions of class specific antisera absorbed with more closely related strains.

The procedures described have been found useful and reliable for the serological classification of gonococcal strains described in recent serological studies by other authors and also for the classification of β lactamase producing strains and strains from patients with disseminated gonococcal infection (16).

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SEROLOGY OF *NEISSERIA GONORRHOEAE* CLASSIFICATION BY CO-AGGLUTINATION

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Sandström E & Danielsson D Serology of *Neisseria gonorrhoeae* Classification by co agglutination
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The co-agglutination (COA) method has been adapted for serological classification of *Neisseria gonorrhoeae*. COA reagents were prepared with selectively absorbed rabbit hyperimmune antibodies against gonococcal (GC) major outer membrane protein (MOMP) serotype strains. Using these reagents the 16 MOMP reference strains could be referred to at least three antigen classes: tentatively named W, J and M. The GC antigens of class W were divided into three groups I, II and III and they were in part sensitive to pronase. The antigens of class J reflected strain specific or serotype reactions, some sensitive and others resistant to proteolytic enzymes. The antigens of class M were sensitive to periodate and resistant to pronase. Strains used in serological studies by other authors were tested. The properties of class W correlated well with those of the so-called micro-immunofluorescence and immunotype systems and class M with those of the so-called endotoxin and acid polysaccharide systems. Strains from three different laboratories could all be grouped by class W and M reagents. Identical strains obtained independently from different laboratories gave very similar reaction patterns with the reagents available. Repeated GC-isolates from patients infected with beta-lactamase producing strains showed stable reactions with class W and J reagents, while there was a time related variation of the class M pattern. We have found that the COA method is rapid, easy and reproducible in the serological classification of *Neisseria gonorrhoeae* and all the 117 GC-strains tested could be classified.

Key words: *Neisseria gonorrhoeae*, co agglutination, serology, classification.

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Ever since the beginning of this century many attempts have been made to achieve a serological classification of *Neisseria gonorrhoeae*. The older works were summarized by Reyn (19) and Nilson

(21). As a result of these studies it was found that the

main difficulty to overcome most of the difficulties in direct agglutination of gonococci. With absorbed antisera against undefined antigens he was able to characterize gonococcal strains by a formula. Further studies along these lines were however discouraged by the finding that gonococci could change serologically on prolonged subculture. Maeland (17) working with indirect haemagglutination (IA)

with periodate sensitive but pronase stable antigens prepared from the endotoxin found antigenic factors of three reference strains best represented by a formula. Apicella (2) purified an acid polysaccharide antigen for use in agar gel diffusion (AGD) and HA studies. Using absorbed antisera he was able to define five serotypes (3). Johnston *et al.* (14) studied by AGD an antigen characterized by major outer membrane protein (MOMP) in polyacrylamide gel electrophoresis and found 16 serotypes in a broad panel of strains. Wang *et al.* (22) applied the so-called micro-immunofluorescence (Micro-IF) to undefined gonococcal antigens with absorbed mouse antisera. With this system three groups with subgroups were observed and a correlation with

We have previously explored the potential use of the co agglutination (COA) method for serological studies of gonococci and the results were strongly correlated to those obtained by indirect immunofluorescence and immunoelectrophoresis (8). Based on the findings by the so-called line rocket immunoelectrophoresis (L RIE) defined reagents could be prepared for COA by selected absorptions. When applied to the 16 MOMP reference strains described by Johnston *et al* (14) the results of COA tests with these absorbed antibodies could be referred to at least three classes of antigens. We suggested that this might form the basis of a serological classification of gonococci according to a multifactor model.

These studies were extended in the present work, and absorbed antibodies were prepared against the MOMP reference strains to identify the three classes of antigens by COA tests. These reagents were then used to classify strains used in serological studies by Macland (17), Geizer (12), Apicella (3), Arko *et al* (4) and Wang *et al* (22) as well as other strains kept at our and other laboratories. Included were also β lactamase producing gonococcal strains isolated from six patients.

Neisseria gonorrhoeae Strains

One hundred and seventeen gonococcal (GC) were included in the present study. They were divided by six groups of reference strains in all 45 and three groups of other defined strains in all 26. Table 1. Forty six strains were isolated from six patients infected with β lactamase producing GC.

Isolates HCA 1-21 were obtained from a male patient (HCA) with multiple sexual exposures in the Far East. They were isolated from urethral and pharyngeal specimens over a 73-day period due to treatment failures. Isolates HCA 1-2 were from urethra, the rest from the pharynx. All isolates except HCA 2 β lactamase.

Isolates BA 1-8 all β lactamase producers obtained from a male patient (BA) with multiple sexual exposures in the Far East. They were isolated over a 14-day period. BA 1 from the urethra, the rest from the pharynx.

Isolates RC 1-3 all β lactamase producers were from a male patient (RC) with a single sexual exposure in the Far East. The strains were isolated from urethral specimens over a 6-day period.

Isolate HA 1 was from the urethra of a male patient (HA) with a history of sexual intercourse in Poland.

Isolate TB 1 was from a male patient (TB) with a

TABLE 1. *Compilation of Neisseria gonorrhoeae* Strains Used in the Present Study

A. Reference Strains Used by Others in Serological Studies

- 1) *Major Outer Membrane Protein (MOMP) Strains* (14) 16 strains obtained from Dr. K. H. Johnston, Houston, Texas, USA. Designations: see Table 2.
- 2) *Micro Immunofluorescence (Micro IF) Reference Strains* (22) eight strains obtained from Drs. K. A. Holmes and J. Knapp, Seattle, Washington, USA. Designations: see Table 3.
- 3) *Immunotyping Reference Strains* (4) eight strains obtained from Dr. K. H. Wang, Atlanta, Georgia, USA. Designations: see Table 3.
- 4) *Endotoxin Reference Strains* (17) three strains obtained from Dr. J. A. Macland, Trondheim, Norway. Designations: see Table 3.
- 5) *Acid Polysaccharide Serogroup Reference Strains* (3) five strains obtained from Dr. M. A. Apicella, Buffalo, N.Y., USA. Designations: see Table 3.
- 6) *Gel Diffusion Reference Strains* (12) five strains obtained from Dr. I. Geizer, Prague, Czechoslovakia. Designations: see Table 3.

B. Other Defined Strains

- 1) *Seattle Strains* nine in all six obtained from Drs. K. A. Holmes and J. Knapp, three from Dr. T. Buchanan, Seattle, Washington, USA. Designations: see Table 4.
- 2) *Oxford Strains* six in all. Four strains described in a previous work (8): one strain 732 from a patient with disseminated gonococcal infection (DGI), one strain P9 obtained from Dr. M. E. Hart, Southampton, England. Designations of the other four strains: see Table 4.
- 3) *Stockholm Strains* 11 in all. Five were described in a previous work (21): designations: see Table 4. Strain 23892 isolated from the knee joint and strains He, Ge, De and Bj from urogenital specimens of five females with DGI. Strain K.1 was isolated from the pharynx of a female with a history of arthralgia two months after intercourse.

C. Strains from Patient Infected with β -Lactamase Producing Strains

Forty six isolates from six patients infected with β -lactamase producing GC were included in the serological tests. H... ..

1 exposure in the Far East. Urethral discharge 14 four days after his return to Stockholm and 15 exposure to patient UL (see below). Clinical and microbiological cure of his β lactamase producing GC 16 after a single oral dose of pivampicillin 2.4 g plus 17 probenecid. Isolates UL 1-12 were from a female patient (UL) 18 was the regular sexual partner of TB (see above) 19 re his trip to the Far East. She had sexual intercourse 20 another partner during TB's stay in the Far East and 21 TB immediately after his return. She also admitted 22 genital practice. Four days later she developed fever 23 C severe pains in her left wrist and both ankles. She 24 had a sore throat. Isolates UL 1-2 were isolated 25 at treatment with pivampicillin 1.4 g three times 26 y for six days. She reported clinical cure but four new 27 ones UL 3-6 were obtained during the last day of 28 imipenem treatment and prior to spectinomycin 29 g ml. Isolates 7-12 were obtained after this 30 treatment. Isolates UL 1-4 were from urogenital 31 cimens the rest from the pharynx.

Isolation

The colony morphology typing medium (CMT) 1 described by Kellogg *et al.* (15) was used throughout the 2 study. The bacteria were grown for 22 h at 37 °C in 5% 3 CO₂ in a humidified incubator. The β lactamase producing 4 cocci were isolated on Oxoid's Columbia agar 5 supplemented with horse serum and blood and identified 6 described elsewhere (7). All isolates were tested for β - 7 lactamase production with chromatogenic cephalosporin 8 disks (Stockholm, Sweden) and for most strains also 9 with the modification of the Escam II a (10) and clover 10 leaf (24) techniques. They were frozen after a minimum 11 subcultures as described previously (8).

Antisera

Antisera against the MOMP strains were obtained 1 from rabbits and absorbed as described previously (21).

Agglutination

Reagent staphylococci were prepared as described 1 elsewhere (16). During the later phase of the study they 2 were obtained as a gift from Dr L. Rudin Pharmacia 3 Diagnostica, Uppsala, Sweden. COA tests were perfor- 4 med with heated whole cells (100 °C for 60 min) as 5 described previously (21) and the reactions were graded 6 negative (-), weak (+), moderate (2+) and strong or 7 very strong (3+).

Treatment with Periodate and Pronase

This was carried out essentially as described by 1 Angelén & Maeland (1). Gonococci were grown for 20- 2 h on CMT medium and harvested in 0.01 M 3 phosphate buffered saline (PBS) pH 7.4, spun down at 4 2500 \times g for 15 min, suspended in a concentration of 5 20 mg/ml wet weight, boiled in a water bath for 1 h 6 and divided into four portions. One portion was spun 7 down and suspended in four times the original volume 8 with 5 mg/ml sodium meta-periodate, kept in a dark 9 bottle overnight at +4 °C, spun down and finally 10 resuspended in the original volume of PBS. A second

portion was treated in the same way as portion one but 1 without periodate. A third portion was spun down and 2 resuspended in the original volume of PBS with 0.4 mg 3 ml⁻¹ pronase, incubated for 4 h at 37 °C and then heated 4 for 5 min at 100 °C to inactivate the pronase. This 5 solution was transparent at the end of the incubation. A 6 fourth portion was treated in the same way as portion 7 three but without pronase.

Inhibition by Sugars

This procedure was modified from Maeland (17) and 1 carried out in two different ways. Initially sensitized 2 reagent staphylococci were spun down and resuspended 3 in the same volume of PBS with a 0.28 M sugar solution 4 to be tested and incubated overnight in the cold before the 5 test. A control without sugar was always included. It 6 was later found that the same results were obtained by 7 simply mixing one drop of sensitized reagent staphylo- 8 cocci with one drop of sugar solution.

66C 0369 D (+) galactose lot 27C 0177 and D (+) 1 glucosamine lot 25C 0286 (Sigma Chemical Co)

RESULTS

1 Description of COA Regents with Reactivity for the Three Suggested Antigen Classes of the MOMP Reference Strains

In a previous study (21) L RIE results guided the 1 absorptions of anti gonococcal antibodies to be used 2 for coating of reagent staphylococci which in COA 3 tests with the MOMP reference strains.

The reactivity patterns of 1 the reagents with each of the strains within these 2 classes are shown in Table 2.

Class II According to the reactions with 1 prepared reagents, strains in this antigen class were 2 divided into three groups: group I comprising 3 strains W 16 B 2 G 7 E 5 R 11 D 4 and V 15; 4 group II strains N 10 H 8 C 3 X 9 S 12 T 13 5 and U 14; and group III strains A 1 and F 6. Strain 6 W 16 reacted with one of the three reagents for W / 7 I and W / II but the antiserum (anti-S 12) used for 8 the W / II group also contained an antibody against a 9 class II antigen. Furthermore antiserum against 10 strain W 16 could be absorbed in a way that made it specific 11 for group W / I but not for group W / II. Thus W 16 12 was referred to W / I.

Class I Seven of the MOMP strains reacted with 1 the reagents in this class. The typical pattern for 2 these strains was that only one or two strains 3 reacted with one of the five class I reagents 4 tentatively designated as indicated in the table

We have previously explored the potential use of the co agglutination (COA) method for serological studies of gonococci and the results were strongly correlated to those obtained by indirect immunofluorescence and immunoelectrophoresis (8). Based on the findings by the so called line rocket immunoelectrophoresis (L-RIE) defined reagents could be prepared for COA by selected absorptions. When applied to the 16 MOMP reference strains described by Johnston *et al* (14) the results of COA tests with these absorbed antibodies could be referred to at least three classes of antigens. We suggested that this might form the basis of a serological classification of gonococci according to a multifactor model.

These studies were extended in the present work, and absorbed antibodies were prepared against the MOMP reference strains to identify the three classes of antigens by COA tests. These reagents were then used to classify strains used in serological studies by Maeland (17), Geizer (12), Apicella (3), Arko *et al* (4) and Wang *et al* (22) as well as other strains kept at our and other laboratories. Included were also β lactamase producing gonococcal strains isolated from six patients.

Neisseria gonorrhoeae Strains

One hundred and seventeen gonococcal (GC) strains were included in the present study. They were represented by six groups of reference strains in all 45, three groups of other defined strains in all 16, 17 designations and sources of these strains are shown in Table 1. Forty six strains were isolated from specimens infected with β lactamase producing GC.

Isolates HCA 1-21 were obtained from a male patient (HCA) with multiple sexual exposures in the Far East. They were isolated from urethral and pharyngeal specimens over a 73-day period due to treatment failures. Isolates HCA 1-2 were from urethra the others from the pharynx. All isolates except HCA 2 produced β lactamase.

Isolates BA 1-8 all β lactamase producers were obtained from a male patient (BA) with multiple sexual exposures in the Far East. They were isolated over a 1 day period. BA 1 from the urethra the rest from the pharynx.

Isolates RC 1-3 all β lactamase producers were from a male patient (RC) with a single sexual exposure in the Far East. The strains were isolated from urethral specimens over a 6 day period.

Isolate HA 1 was from the urethra of a male patient (HA) with a history of sexual intercourse in Poland.

Isolate TB 1 was from a male patient (TB) with a history of sexual intercourse in Poland.

TABLE 1. *Compilation of Neisseria gonorrhoeae* Strains Used in the Present Study

A. Reference Strains Used by Others in Serological Studies

- 1) *Major Outer Membrane Protein (MOMP) Strains* (14) 16 strains obtained from Dr A. H. Johnston, Houston, Texas, USA. Designations see Table 2.
- 2) *Micro Immunofluorescence (Micro IF) Reference Strains* (22) eight strains obtained from Drs A. A. Holmes and J. Knapp, Seattle, Washington, USA. Designations see Table 3.
- 3) *Immunotyping Reference Strains* (4) eight strains obtained from Dr A. H. Wang, Atlanta, Georgia, USA. Designations see Table 3.
- 4) *Endotoxin Reference Strains* (17) three strains obtained from Dr J. A. Maeland, Trondheim, Norway. Designations see Table 3.
- 5) *Acid Polysaccharide Serogroup Reference Strains* (3) five strains obtained from Dr M. A. Apicella, Buffalo, NY, USA. Designations see Table 3.
- 6) *Gel Diffusion Reference Strains* (12) five strains obtained from Dr I. Geizer, Prague, Czechoslovakia. Designations see Table 3.

B. Other Defined Strains

- 1) *Seattle Strains* nine in all six obtained from Drs A. A. Holmes and J. Knapp, three from Dr T. W. Richmond, Seattle, Washington, USA. Designations see Table 4.
- 2) *Other Strains* six in all. Four strains described in a previous work (8), one strain 732 from a patient with urethritis, and one strain 733 from a patient with urethritis, both from Southampton.
- 3) *Isolate 1892* isolated from the knee joint and strains He, Ge, De and Bj from urogenital specimens of five females with DGC. Strain 1892 was isolated from the pharynx of a female with a history of arthralgia two months after intercourse.

C. Strains from Patient Infected with β -Lactamase Producing Strains

Forty six isolates from six patients infected with β -lactamase producing GC were included in the serological tests. History of these patients see text.

il exposures in the Far East Urethral discharge
 11 four days after his return to Stockholm and
 al exposure to patient UL (see below) Clinical and
 etiological cure of his β -lactamase producing GC
 n after a single oral dose of pivampicillin 2.4 g plus
 g probenecid

isolates UL 1-12 were from a female patient (UL)
 1 was the regular sexual partner of TB (see above)
 re his trip to the Far East. She had sexual intercourse
 1 another partner during TB's stay in the Far East and
 b TB immediately after his return. She also admitted
 genital practice. Four days later she developed fever
 °C, severe pains in her left wrist and both ankles. She
 1 had a sore throat. Isolates UL 1-2 were isolated
 or to treatment with pivampicillin 1.4 g three times
 for six days. She reported clinical cure but four new
 lates UL 3-6 were obtained during the last day of
 ampicillin treatment and prior to spectinomycin
 1 g im. Isolates 7-12 were obtained after this
 atment. Isolates UL 1-4 were from urogenital
 scimens the rest from the pharynx

ulture

The colony morphology typing medium (CMT)
 scribed by Kellogg *et al.* (15) was used throughout the
 ydy. The bacteria were grown for 22 h at 37 °C in 5%
 O₂ in a humidified incubator. The β -lactamase producing
 inococci were isolated on Oxoids Columbia agar
 1plemented with horse serum and blood and identified
 described elsewhere (7). All isolates were tested for β -
 ctamase production with chromatogenic cephalosporin
 iodisk (Stockholm, Sweden) and for most strains also
 with the modification of the Escam IIIa (10) and clover
 af (24) techniques. They were frozen after a minimum
 f subcultures as described previously (8).

ntisera

Anusera against the MOMP strains were obtained
 om rabbits and absorbed as described previously (21).

o agglutination

Reagent staphylococci were prepared as described
 elsewhere (16). D₅₀ =

v =

) =

n =

less than previously (21) and the reactions were graded
 negative (-), weak (+), moderate (2+) and strong or
 ery strong (3+).

Treatment with Periodate and Pronase

This was carried out essentially as described by
 Angelens & Maeland (1). Gonococci were grown for 20-
 22 h on CMT medium and harvested in 0.01 M
 phosphate buffered saline (PBS) pH 7.4, spun down at
 2,500 \times g for 15 min, suspended at a concentration of
 20 mg/ml wet weight, boiled in a water bath for 1 h
 and divided into four portions. One portion was spun
 down and suspended in four times the original volume
 with 5 mg/ml 1% sodium meta-periodate, kept in a dark
 bottle overnight at +4 °C, spun down and finally
 resuspended in the original volume of PBS. A second

portion was treated in the same way as portion one but
 without periodate. A third portion was spun down and
 resuspended in the original volume of PBS with 0.4 mg
 ml⁻¹ pronase, incubated for 4 h at 37 °C and then heated
 for 5 min at 100 °C to inactivate the pronase. This
 solution was transparent at the end of the incubation. A
 fourth portion was treated in the same way as portion
 three but without pronase.

Inhibition by Sugars

This procedure was modified from Maeland (17) and
 carried out in two different ways. Initially sensitized
 reagent staphylococci were spun down and resuspended
 in the same volume of PBS with a 0.28 M sugar solution
 to be tested and incubated overnight in the cold before the
 test. A control without sugar was always included. It
 was later found that the same results were obtained by

lot 117C 0046 α -lactose lot 14C 1650 β -lactose lot
 66C 0369 D (+) galactose lot 27C 0177 and M (+)
 glucosamine lot 25C-0286 (Sigma Chemical Co)

RESULTS

1 Description of COA Regents with Reactivity for the Three Suggested Antigen Classes of the MOMP Reference Strains

In a previous study (21) L-RIE results guided the
 absorptions of anti-gonococcal antibodies to be used
 for coating of reagent staphylococci which in COA
 tests with the MOMP reference strains of Johnston
et al. (14) gave reactions that fulfilled the criteria for
 each of the three suggested antigen classes tentatively
 called W, I and M. The reactivity patterns of the
 reagents with each of the strains within these
 classes are shown in Table 2.

Class W According to the reactions with
 prepared reagents, strains in this antigen class were
 divided into three groups: group I comprising
 strains W 16, B 2, G 7, E 5, R 11, D 4 and V 15;
 group II strains N 10, H 8, C 3, X 9, S 12, T 13
 and U 14; and group III strains A 1 and F 6. Strain
 W 16 reacted with one of the three reagents for W/
 I and W/II, but the antiserum (anti M 12) used for
 the W/II group also contained an antibody against a
 class M antigen. Furthermore, antiserum against
 strain W 16 could be absorbed to make it specific
 for group W/I but not for group W/II. Thus W 16
 was referred to W/I.

Class I Seven of the MOMP strains reacted with
 the reagents in this class. The typical pattern for
 these strains was that only one or two strains
 reacted with one of the five class I reagents
 tentatively designated as indicated in the table.

TABLE 2 *Co agglutination Pattern of the MOMP Reference Strains with Reagents for the Three Suggested Antigen Classes Tentatively named W, J and M*

Reagent staphylococci coated with absorbed anti GC antibodies	Co agglutination reactions with MOMP reference strains																Suggested antigen class pattern
	W 16	B 2	G 7	E 5	R 11	D 4	V 15	N 10	H 8	C 3	Y 9	S 12	T 13	U 14	A 1	F 6	
Anti E 5 abs w N 10	-	3+	3+	3+	3+	1+	2+	-	-	-	-	-	-	-	-	-	W/I
Anti D 4 abs w C 3	2+	1+	1+	1+	3+	3+	3+	-	-	-	-	-	-	-	-	-	
Anti V 15 abs w C 3	-	-	-	-	2+	2+	3+	-	-	-	-	-	-	-	-	-	
Anti N 10 abs w D 4	-	-	-	-	-	-	-	3+	3+	3+	3+	3+	-	-	-	-	
Anti S 12 abs w A 1	2+	-	-	-	-	-	-	3+	3+	3+	3+	3+	3+	3+	-	-	W/II
Anti U 14 abs w R 11	-	-	-	-	-	-	-	2+	2+	2+	2+	-	2+	2+	-	-	
Anti F 6 abs w B 2 & U 14	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2+	3+	W/III
Anti D 4 abs w B 2 & C 3	-	-	-	-	-	3+	3+	-	-	-	-	-	-	-	-	-	
Anti U 14 abs w C 3	-	-	-	-	-	-	-	-	-	-	-	-	-	3+	-	-	
Anti Y 9 abs w A 1 & B 2	-	-	-	-	-	-	-	-	-	-	2+	-	3+	3+	-	-	
Anti S 12 abs w Y 9	-	-	-	-	-	-	-	-	-	-	-	3+	-	-	-	1+	J/4 J/14 J/9 J/12 J/6
Anti F 6 abs w A 1 & B 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	3+	
Anti C 3 abs w A 1	-	2+	3+	2+	3+	3+	3+	2+	3+	3+	-	-	-	-	-	-	
Anti D 4 abs w B 2	-	-	-	1+	3+	3+	3+	-	-	-	-	-	-	-	-	3+	
Anti C 3 abs w N 10	-	-	-	-	-	-	-	-	-	3+	-	-	-	-	-	-	M/a M/b M/c M/d M/e M/f M/g
Anti Y 9 abs w C 3	1+	2+	2+	-	-	-	-	-	3+	-	3+	-	3+	3+	3+	-	
Anti S 12 abs w H 8	2+	-	-	-	-	-	-	-	-	-	3+	-	-	-	-	-	
Anti E 5 abs w R 11	-	2+	3+	3+	-	-	2+	2+	+	-	-	-	-	-	-	-	
Anti A 1 abs w F 6	-	-	-	-	-	1+	1+	-	-	-	-	-	-	-	2+	-	

antigens responsible for these reactions thus seem to reflect "strain" or "serotype" specificity

Class M The typical patterns for the reagents in this class were reactions with one or more strains in both W/I and W/II, and for some reagents for strains in all three W groups. The seven reagents of class M were tentatively designated as M/a-g

Based on the suggested antigen class pattern a particular gonococcal strain for example strain 24 could be classified as W/I J/4, M/a, b. Correspondingly, strain X-9 could be classified as W/II J/9 M/d, and strain A-1 as W/III M/d, g

1) Effect of Treatment with Pronase and Periodate

Some of the antigens of MOMP strains responsible for Class W reactions were sensitive to pronase and all were resistant to periodate. The antigens of the two MOMP strains X-9 and S-12, responsible for J/9 and I/12 reactions, were sensitive to pronase and resistant to periodate. The antigens of the other strains in this class were resistant to both pronase and periodate. The class M reactive antigens of the MOMP strains were sensitive to periodate and resistant to pronase which was used as an additional criterion for this class

3) COA Patterns of Selected Reference Strains with Reagents for the Three Antigen Classes

Twenty nine selected gonococcal strains (for details of source etc see Table 1) were tested by reagent staphylococci for the three tentative antigen classes in Table 2. The antigen class patterns are shown in Table 3

Micro-IF reference strains (Wang et al (22)) The reference strains for the Micro-IF groups A, B and C reacted with group W/I, W/II and W/III respectively. In addition two of the group B reference strains reacted with the W/III reagent and one group C strain with one of W/II reagents. All group B and C strains but none of the group A strains reacted with class J reagents. All eight reference strains reacted with class M reagents in various combinations

According to these findings the antigen class pattern of strain A₂ could be described as W/I M/a b c strain B₃ as W/II J/12, 14 M/e and strain C₁ as W/III J/6 M/a d

Immunotype reference strains (Arko et al (4))

Ehrlich of the ...

Strain ... which antiserum was also found to contain a class J antibody reacting with this strain. Strain 2686 reacted with the W/III and with one of the W/II reagents and strain WJ1/1 with another W/II and the W/III reagent. These three strains gave

protection only to themselves in guinea-pig chambers, while the other five strains cross reacted in various combinations (4). The close correspondence observed between the class W of COA and the immunotyping system is in agreement with that observed for Micro-IF and the latter system (18)

Four of the strains reacted with class J reagents and all eight with class M reagents in various combinations

Endotoxin reference strains (Maeland (17)) The three strains reacted with W/II, III, W/II and W/I, and only one gave a class J reaction

Strain 8551 reacted with factors M/a, b, c, strain V with M/a, d, e and strain VII with M/d. All of these reactions were sensitive to periodate and resistant to pronase. The M/a reactions for strains

galactose, lactose or glucosamine (Table 3). α - and β -lactose had the same blocking effect. The combinations of COA reactions of these three strains with our M/a, b, c, d reagents, their sensitivity to periodate, resistance to pronase and inhibition by sugars correspond to the characteristics of the antigenic factors a₆, a₃ and a₁ described by Maeland (16, 17). The M/e reaction was too weak to be reliably tested in the sugar inhibition tests, but other characteristics corresponded to factor a₂ reported by Maeland

Acid polysaccharide serogroup reference strains (Apicella (3)) The five strains all reacted with W/II and in addition some with W/I, III

Reaction of this strain was destroyed by pronase and thus not a true class M reaction. Strain 4505/Gc₃ reacted with M/a, b, d, e, but the reactions M/d and M/e were destroyed by pronase and resistant to periodate, and were thus not true M reactions

and the results of sugar inhibition tests are shown in Table 5. The sugar inhibition pattern for each M factor corresponds to that found with the

W1

(3)

Agglutination serotype reference strains (Geizer (12)) Two strains reacted with W/I and three with W/II. One strain reacted with a class J reagent and all strains with class M reagents in various

Phage Classes W, J and M React as Graded as Described in Materials and Methods

Wx/h	Wll/l	Endotoxin ref strains ^{c)}			Acid polysaccharide ref strains ^{d)}					Gel diffusion serotype strains ^{e)}				
		8551	V	VII	1342 GC ₁	1291 GC ₂	4501 GC ₃	8551 GC ₄	P102 GC ₅	822/A	554/B	986/C	390/D	509/E
-	-	-	-	2+	-	-	-	-	-	3+	-	3+	-	-
-	-	-	-	-	-	-	-	-	-	-	-	1+	-	-
-	-	-	-	-	-	-	-	-	-	3+	-	3+	-	-
3+	3+	-	3+	-	3+	3+	3+	-	3+	-	3+	-	3+	3+
3+	-	-	3+	-	3+	3+	3+	-	3+	-	3+	-	-	1+
1+	1+	2+	-	-	3+	2+	2+	2+	2+	-	3+	-	1+	1+
2+	2+	3+	-	-	-	-	-	3+	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	3+	-	-	2+	-	-	-
-	-	-	-	-	1+	-	1+	-	-	-	-	-	-	-
-	-	3+	-	-	2+	1+	2+	-	3+	-	-	-	-	-
-	-	-	-	-	-	-	-	3+	-	-	-	-	-	-
3+	3+	3+	2+	-	3+	2+	3+	3+	3+	3+	3+	3+	3+	3+
1+	1+	3+	-	-	3+	-	3+	3+	2+	2+	3+	2+	-	-
-	-	3+	-	-	-	-	-	3+	-	-	3+	-	-	-
3+	3+	-	3+	3+	-	3+	2+	-	-	3+	-	3+	3+	-
-	-	-	2+	-	-	1+	3+	-	2+	1+	1+	-	2+	2+
-	-	-	-	-	3+	-	-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-	-	-	-	-	-	3+	-

Osborn strains					Stockholm strains										
III	SO ₄	505	712	P9	14984	15057	15000	11376	14190	23897	Hc	Ge	B ₁	h1	De
3	3+		3	1	3+			3+	3+	3+	3+	3+	1+	3+	2+
3+	3+		3+		2+	3+		3+	2+	2+	2+	3+			-
3	3+		3+					1+	2+		3+	3+	1+	2+	2+
		1		3+		3+	3								-
		3+		3+		3+	3+								-
		3+		3+		3+	3								-
					1										
1				2+		3+									
	1			1+											
				3+		3+									
3+	3+			2+	1+	3+	3+	3+		3+	3+	3+	3+	3+	
3	1			1	1+	3+	3+	3+		3+	3+	3+	3+	3+	
	1+			1+				3+		3+	3+	3+	3+	3+	
3	3+	3		3+		1+	3+		3+	3+	3+	3+	2+	3+	
2					2+	1+			3+			3+	3+	3+	
						1+	3+	3+							1+
				1+									3+		

TABLE 3 *Co agglutination Patterns of 29 Selected Reference Strains with Reagents for the Three Tests*

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	Micro IF reference strains ^{a)}								Immunotype refer		
		4286 (A ₁)	1859 (A ₂)	1567 (A ₃)	5293 (B ₁)	5288 (B ₂)	5001 (B ₃)	5016 (C ₁)	1955 (C ₂)	A/a	C/c	G/d N
Anti E 5 abs w N 10	W/I	3+	3+	3+	-	-	-	-	-	-	-	3+
Anti D 4 abs w C 3		3+	3+	3+	-	-	-	-	-	-	-	3+
Anti V 15 abs w C 3		3+	3+	3+	-	-	-	-	-	-	-	3+
Anti N 10 abs w D 4		-	-	-	-	3+	2+	-	-	3+	3+	-
Anti S 12 abs w A 1	W/II	-	-	-	-	3+	3+	3+	-	3+	-	3+
Anti U 14 abs w B 11		-	-	-	3+	3+	2+	-	-	2+	-	3+
Anti F 6 abs B 2&U 14	W/III	-	-	-	3+	3+	-	3+	3+	-	1+	-
Anti D 4 abs w B 2&C 3	J/4	-	-	-	-	-	-	-	-	-	-	-
Anti U 14 abs w C 3	J/14	-	-	-	1+	1+	2+	-	-	-	-	-
Anti X 9 abs w A 1&B 2	J/9	-	-	-	-	-	-	-	-	-	-	-
Anti S 12 abs w X 9	J/12	-	-	-	-	-	2+	-	-	2+	-	3+
Anti F 6 abs w A 1&B 2	J/6	-	-	-	-	2+	-	3+	3+	-	-	-
Anti C 3 abs w A 1	M/a	3+	3+	3+	-	3+	1+	2+	3+	3+	3+	3+
Anti D 4 abs w B 2	M/b	-	3+	3+	1+	3+	-	-	-	-	-	1+
Anti C 3 abs w N 10	M/c	-	2+	2+	-	3+	-	-	-	-	-	1
Anti X 9 abs w C 3	M/d	-	-	-	3+	3+	1+	3+	-	3+	-	3+
Anti S 12 abs H 8	M/e	1+	-	-	2+	1+	3+	-	-	2+	-	3+
Anti E 5 abs w R 11	M/f	3+	-	3+	-	-	-	-	3+	1+	-	3
Anti A 1 abs w F 6	M/g	-	-	-	3+	1+	-	1+	-	-	-	-

a) Wang *et al* see ref (22) b) Arko *et al* see ref (4) c) Maeland see ref (17) d) Apicella see ref (3) e) Gei *et al* seeTABLE 4 *Co agglutination Patterns of 26 Defined Gonococcal Strains Obtained from Seattle Örebro and Stockholm*

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	Seattle strains								
		5029	5010	5030	5064	6218	F62	Black	C 13	
Anti E 5 abs w N 10	W/I	3+	-	3+	3+	-	-	-	-	-
Anti D 4 abs w C 3		3+	3+	3+	-	-	-	-	-	-
Anti V 15 abs w C 3		2+	-	3+	-	-	-	-	-	-
Anti N 10 abs w D 4	W/II	-	-	-	-	-	-	3+	-	-
Anti S 12 abs w A 1		-	-	-	-	3+	-	3+	3+	-
Anti U 14 abs w R 11		-	-	-	-	3+	2+	2+	3+	-
Anti F 6 abs w B 2 & U 14	W/III	-	-	-	-	-	3+	-	-	-
Anti D 4 abs w B 2 & C 3	J/4	-	3+	-	-	-	-	-	-	-
Anti U 14 abs w C 3	J/14	-	-	-	-	3+	1+	3+	3+	-
Anti X 9 abs w A 1 & B 2	J/9	-	-	-	-	-	-	-	-	-
Anti S 12 abs w X 9	J/12	-	-	-	-	-	-	2+	-	-
Anti F 6 abs w A 1 & B 2	J/6	-	-	-	-	-	-	-	-	-
Anti C 3 abs w A 1	M/a	3+	3+	3+	3+	-	-	3+	-	-
Anti D 4 abs w B 2	M/b	2+	3+	3+	2+	-	1+	3+	-	-
Anti C 3 abs w N 10	M/c	2+	-	-	1+	-	-	-	-	-
Anti X 9 abs w C 3	M/d	3+	-	-	2+	3+	3+	1+	2+	-
Anti S 12 abs w H 8	M/e	2+	-	3+	-	1+	1+	3+	-	-
Anti E 5 abs w R 11	M/f	-	3+	-	3+	-	-	3+	-	-
Anti A 1 abs w F 6	M/g	2+	1+	1+	-	-	1+	-	-	-

TABLE 5 Co-agglutination Patterns of the «Endotoxin» and «Acid Polysaccharide» Reference Strains with Class Reagents Effect of Treatment with Pronase and Periodate Blocking of the Reactions with Sugars

Reagent staphylococci coated with absorbed anti-GC antibodies	Tentative antigen class pattern	Endotoxin reference strains			Acid polysaccharide reference strains			
		8551	V	VII	1342 GC ₁	1291 GC ₂	4505 GC ₃	8551 GC ₄
Anti C-3 abs w A-1	M/a	r/sb(L)	r/s(L)	-	r/s(L)	s/s	r/s(GL)	r/s(L)
Anti D 4 abs w B-2	M/b	r/s(L)	-	-	r/s(GL)	-	r/s(GL)	r/s(L)
Anti C-3 abs w N-10	M/c	r/s(GL)	-	-	-	-	-	r/s(GL)
Anti X-9 abs w C-3	M/d	-	r/s(NS)	r/s(NS)	-	r/s(NS)	s/r	-
Anti S-12 abs w H-8	M/e	-	r/s(nd)	-	-	r/s(nd)	s/r	-
Anti E-5 abs w R-11	M/f	-	-	-	r/s(NS)	-	-	-
Anti A-1 abs w F-6	M/g	-	-	-	-	-	-	-

a) Effect of treatment with pronase r = resistant s = sensitive

b) Effect of treatment with periodate r = resistant, s = sensitive

c) Blocking of reactions with sugars

L = blocking of the reaction with lactose

GL = blocking of the reaction with galactose and lactose

NS = no blocking of the reaction with glucose, galactose, lactose or glucosamine

nd = not done

combinations Strains 882/A and 986/C were very similar, as were 390/D and 509/E. All strains were used by Geizer to absorb all heterologous antisera. Unique reactions are found only for 554/B in class J and M for strain 390/D in class M.

TABLE 6 Co-agglutination Patterns of β -Lactamase Producing Gonococcal Strains Isolated from Six Patients Th

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	Patient HCA isolate No						
		1	2	3 14	15	16	17	18
Anti E 5 abs w N 10	W/I	-	-	-	-	-	-	-
Anti D 4 abs w C 3		-	-	-	-	-	-	-
Anti V 15 abs w C 3		-	-	-	-	-	-	-
Anti N 10 abs w D 4		-	-	-	-	-	-	-
Anti S 12 abs w A 1	W/II	3+	3+	3+	3+	3+	3+	3+
Anti U 14 abs w R 11		-	-	-	-	-	-	-
Anti F 6 abs w H 2&L 14	W/III	-	-	-	-	-	-	-
Anti D 4 abs w B 2&C 3	J/4	-	-	-	-	-	-	-
Anti U 14 abs w C 3	J/14	-	-	-	-	-	-	-
Anti X 9 abs w A 1&B 2	J/9	-	-	-	-	-	-	-
Anti S 12 abs w X 9	J/12	2+	2+	3+	3+	3+	3+	3+
Anti F 6 abs w A-1&B 2	J/6	-	-	-	-	-	-	-
Anti C 3 abs w A 1	M/a	3+	3+	3+	3+	-	-	2+
Anti D 4 abs w B 2	M/b	3+	-	3+	-	-	-	-
Anti C-3 abs w N 10	M/c	3+	-	2+	-	-	-	-
Anti X 9 abs w C 3	M/d	-	-	-	3+	3+	3+	3+
Anti S 12 abs w H 8	M/e	-	-	-	-	-	-	-
Anti E 5 abs w R 11	M/f	-	3+	-	-	-	2+	3+
Anti A-1 abs w F 6	M/g	-	-	-	-	-	-	-

a) Isolate No 5 gave a 2+ reaction

b) Isolate No 13 gave a 2+ reaction

Dr Wong and as A-1 (14) from Dr Johnston (Table 1) The COA reactions obtained with A-1 were also found for 2686/g which had one additional reaction in classes W, J and M. It will be seen that the complete reaction pattern of this strain was very similar to that of the two F62 strains from Seattle.

The close relationship between NRL 5293 (F62) and 2686/g and their difference from the KF/f (F62) was also observed by Micro IF (18).

6) COA Patterns with Strains from Patients with Disseminated Gonococcal Infection

Strain WJ 1/1 (Table 3) was reported to be isolated from a heart valve (4). Strains 670, 732, 23892, De He, Ge and Bj were all isolated from patients with DGI. Strain NRL 7122 is used for the isolation of a principal outer membrane protein found in most strains causing DGI (13). All these strains are reactive with W/I except WJ 1/1 which reacts with W/II. This is of interest, since DGI strains are referred to groups A₂ and A₃ in the Micro-IF (22), and strain WJ 1/1 is reported to react with group B (18).

7) COA Patterns with β -Lactamase Producing Strains

From six patients, without possibility of reinfection, β -lactamase producing gonococci were isolated during the period of the study. The serological patterns of 46 isolates from these patients are shown in Table 6.

Three patients (HCA, BA and RC) infected January-March 1978 in the Far East, had the same W/II pattern which was different from that of a patient (HA) infected in Poland, and a patient (TB) infected in the Far East one year later.

Twenty-one isolates were obtained from patient HCA over a 73 day period due to repeated treatment failures of his pharyngeal gonorrhoea. All these isolates had the same class W and J reactions. However, there were obvious variations in the class M patterns. The first urethral isolate prior to pivampicillin produced β -lactamase while the urethral isolate after treatment did not. These two isolates had different class M patterns. The β -lactamase producing pharyngeal isolate HCA 3 had the same class M pattern as the initial urethral isolate and this was also true for the subsequent 11 pharyngeal isolates. With isolate HCA 15, after 53 days a continuous change was noted in the class M reactions which could not be correlated with treatment attempts.

The different class J and M reactions in the pharyngeal isolates BA 2 and 4 represented strains with an antibiogram markedly different from the urethral isolate BA 1 and the pharyngeal isolates

BA 3, 5 and 6. However, isolates BA 2 and 3 were obtained at the same time from different pharyngeal swabs as BA 4 and 5. Isolates BA 7 and 8 were obtained at different times had antibiograms of the BA 1, BA 2 types, respectively, but differed in the class M way from all previous isolates in the class reactions. Thus, in these two patients with suspicion of an infection with more than one strain, the class M reaction patterns showed variations in repeated isolates.

The Swedish consort UL of TB was found to have β -lactamase producing gonococci with same class W and M pattern as TB. The reactivity of UL 3-6 with the second W/I was confirmed with absorbed antibodies. This change occurred during treatment with pivampicillin which cured symptoms. The initial COA pattern recurred in 7-12 after treatment with spectinomycin.

DISCUSSION

In the present work we have extended studies of the serologic classification of *Neisseria gonorrhoeae* by coagglutination (8, 21) with closer examination of the three classes of antigen that we proposed previously (21) and tentatively named W, J and M. Antigen class W was found to correspond well with the three groups in the Micro IF system described by Wang *et al.* (22), and the immunotyping system of Arko *et al.* (19). Antigen class J conforms to some extent with MOMP serotype system of Johnston *et al.* (1) while antigen class M is closely related to endotoxin and acid polysaccharide systems (Maeland (17) and Apicella (3)). We have found agglutination to be a rapid, easy and reproducible method for classification of gonococci along titration lines. Moreover, it can be used to study sensitivity of antigens to proteolytic enzymes and oxidizing agents, and to study the inhibition of agglutination by the reactivity of polysaccharide antigens.

Most of the MOMP reference strains were tested in the Micro IF system by Wang (personal communication). He found that strains B 2, G 7, E 5 and R 11 belonged to group A, while strains W-16 C and V-15 could not be classified. Strains N-10, H C-3, X-9 and A-1 belonged to group B while strain F 6 was placed in group C. Strain T-13 was found to be intermediate between groups B and C but was finally placed in group B. Most of the MOMP reference strains were thus classified analogously in Micro IF and COA, with the exception of strain A-1 which was referred to W/III and group

W 16 D 4 and V 15 which we placed in W/I. Our failure to place strain A 1 in group W/II instead of group W/III could be due to the cross reactivity of the Micro-IF group II and C reference strains seen with the available COA reagents. Wang's failure to classify strains W 16 D 4 and V 15 by Micro-IF could be due to the fact that strain NRL-5029 was used as absorbing strain by Wang *et al* (22) and this strain reacted with our W/I reagents.

COA of the immunotyping strains of Arko *et al* (14) gave a pattern similar to that obtained with Micro-IF by Mark & Wang (18). Furthermore COA tests discriminated between the strains that gave protection only for the homologous strain in guinea pig chamber model and between the ones that gave cross protection (4). It was also of

reagents in the COA tests like group A strains. Our findings in this and a previous work (21) indicated that there was a sharp antigenic demarcation between the W/I and W/II groups. However, within the groups there seemed to be a gradual set of relatedness as judged on the basis of glutinating properties and groups of closely related strains absorbed on the ansera of each other. We therefore suggest that the group W/I and

though highly protected against proteolytic enzymes which is analogous to the meningococcus major outer membrane proteins (11). We suggest that each structure may vary to a limited extent and carry multiple antigenic sites. The variations within W/I would then be represented by strains W 16 D 2 G 7 E 5 R 11 D 4 V 15. Correspondingly the variations within W/II would be represented by 10 H 8 C 3 X 9 S 12 T 13 U 14. The strains within W/III might be included in W/II. However, rational subgrouping of the class W groups must await biochemical characterization of these antigens.

Some of the class J reactions might well fit into class W, for example the reactions of D 4 and V 15 (13) and U 14 and E 6 which are pronase stable. Many of the class J reactions have also been found to be dependent on colony morphology (9). The possible relationship of class J to the MOVP serotype system of Johnston *et al* (14) is therefore not clear at present.

The class M reactions were found to be correlated with both the endotoxin (17) and the acid polysaccharide systems (3). The formulas presented in this work for other strains should however be taken

with caution since some of the reagents had an additional activity against pronase sensitive antigens. It was of interest to note that strain 1342 (GC₁) used for absorptions by Apicella (3) contained more than one M factor. Sadoff *et al* (20) showed by solid phase radioimmunoassay that LPS and the acid polysaccharide of GC₁ had a broad reactivity. Noteworthy was also the broad reactivity within class M of strain NRL-5029 which was considered to be an antigenically unique gonococcus strain by Wang *et al* (22). Repeated isolates of β lactamase producing gonococci showed stable reactions for class W and class J while there were considerable variations in the class M patterns.

The nomenclature suggested in this paper should be considered to be tentative. There will certainly be a need to harmonize the Micro-IF groups and the COA class W groups when more information is obtained about these antigens. We have avoided capital letters since these are used for the capsular antigens of *N meningitidis*. For class J we have adopted an operational classification with arabic numerals depending on the reaction pattern seen with the MOVP reference strains. However as suggested above this class may turn out to be very heterogeneous. Arabic numerals for protein antigens analogous to those of *N meningitidis* would still be useful. Finally we have used small letters for the class M factors.

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SEROLOGY OF *NEISSERIA GONORRHOEAE* DEMONSTRATION BY CO-AGGLUTINATION AND IMMUNOELECTROPHORESIS OF ANTIGENIC DIFFERENCES ASSOCIATED WITH COLOUR/OPACITY COLONIAL VARIANTS

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Key words: *Neisseria gonorrhoeae* co-agglutination immunoelectrophoresis serology

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TABLE 1 COA Reactions Obtained with MOMP and Micro-IF Reference Strains and with GC

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	MOMP reference strains										
		W-16	B 2	G-7	E-5	R-11	D 4	V-15	N 10	H 8	C 3	1
Anti F-5 abs w N-10	W/I	-	3+	3+	3+	3+	1+	2+	-	-	-	-
Anti D 4 abs w C-3		2+	1+	1+	1+	3+	3+	3+	-	-	-	-
Anti V-15 abs w C-3		-	-	-	-	2+	2+	3+	-	-	-	-
Anti N-10 abs w D 4	W/II	-	-	-	-	-	-	-	3+	3+	3+	3
Anti S-12 abs w A-1		-	-	-	-	-	-	-	3+	3+	3+	3
Anti U-14 abs w R-11	W/III	-	-	-	-	-	-	-	2+	2+	2+	2
Anti F 11 abs w B-2 & U-14		-	-	-	-	-	-	-	-	-	-	-
Anti 670 Op abs w P9 Op	W/I	-	2+	2+	2+	3+	3+	1+	-	-	-	-
Anti 670 Op abs w P9 Tr		-	2+	2+	2+	3+	3+	1+	-	-	-	-
Anti 670 Tr abs w P9 Op		1+	1+	1+	2+	2+	1+	-	-	-	-	-
Anti 670 Tr abs w P9 Tr		-	2+	1+	1+	2+	2+	-	-	-	-	-
Anti 670 Op abs w SOA Op/Tr	J/12	-	-	-	-	-	-	-	-	-	-	-
Anti 670 Op abs w 670 Tr		-	-	-	-	-	-	-	-	-	-	-
Anti SOA Op abs w P9 Op/Tr	W/I	3+	3+	3+	3+	3+	3+	3+	-	-	-	-
Anti SOA Tr abs w P9 Op		3+	3+	3+	3+	3+	2+	1+	-	-	-	-
Anti SOA Tr abs w P9 Tr		1+	3+	3+	1+	3+	2+	-	-	-	-	-
Anti P9 Op abs w 670 Tr	W/II	-	2+	-	-	2+	-	-	2+	2+	1+	1
Anti P9 Tr abs w SOA Op		-	-	-	-	-	-	-	2+	2+	2+	1
Anti P9 Tr abs w SOA Tr		-	-	-	-	-	-	-	2+	3+	3+	3

a) Op = Opaque b) Tr = Transparent c) = reaction resistant to pronase d) = reaction sensitive to pronase

In a previous work minor variations in the COA reactions were noted with some strains (3). In the present study it is shown that such variations can be ascribed to antigenic differences associated with colour/opacity variants of isogenic strains. The reproducibility of the COA method for serological classification of *Neisseria gonorrhoeae* is investigated as well as the incidence of reactions dependent on colony morphology variants.

MATERIAL AND METHODS

Neisseria gonorrhoeae Strains and Culture

Eighty five gonococcal (GC) strains were included in the present study, 27 of which were represented by the 16 major outer membrane protein (MOMP) serotype strains (designations see Table 1) described by Johnston *et al.* (14) and the 11 reference strains of GC (3, 10). Fifty-eight strains were unselected clinical isolates from male and female patients attending the outpatient

departments for venereal diseases at Central City Hospital, Örebro and at Södersjukhuset, Stockholm, Sweden (34 of the strains were from two or urogenital and/or rectal sites in 14 patients). A strains were isolated and identified as described previously (1, 2).

The GC organisms for use in COA tests were cultured for 18–22 h at 35–36 °C in candle jars or in a CO₂ thermostat on the colony morphology typing medium (CMT) described by Kellogg *et al.* (7). The α morphology of the MOMP strains has been described previously (9). The Micro IF strains consisted of of T2 colony variants except A₃ and B₁ which had 1 accepted as colony variants resembling T3. Opaque transparent variants of colony morphology type T2 selected according to criteria described by Swanson¹. These T2 colonies showed distinct edges by microscopic. Those which were dark brown or very brown by diffusing substage mirror were opaque by polished mirror and those which were light or brown by diffusing mirror were transparent (lit. polished mirror). Both opaque and transparent colonies showed double highlights as described by I *et al.* (8) when illuminated from above at an angle of

variants of Three Isogenic Strains Reactions Graded as Described in Material and Methods

U 14 A 1 F 6	Col / opac. colonial variants						Micro IF reference strains							
	670 T2		SOA T2		P9 T2		4286	1859	1567	5293	5288	5001	5016	1955
	Op	Tr	Op	Tr	Op	Tr	(A ₁)	(A ₂)	(A ₃)	(B ₁)	(B ₂)	(B ₃)	(C ₁)	(C ₂)
-	3+	3+	3+	3+	-	-	3+	3+	3+	-	-	-	-	-
-	3+	3+	3+	3+	-	-	3+	3+	3+	-	-	-	-	-
-	2+	3+	3+	3+	-	-	3+	3+	3+	-	-	-	-	-
-	-	-	-	-	3+	3+	-	-	-	-	3+	2+	-	-
3+	-	-	-	-	3+	3+	-	-	-	-	3+	3+	3+	-
2+	-	-	-	-	3+	3+	-	-	-	3+	3+	3+	-	-
-	3+	3+	-	-	-	-	-	-	-	3+	3+	-	3+	3+
-	-	-	3+	1+	3+	2+	2+	2+	-	-	-	-	-	-
-	3+	2+	3+	3+	-	-	3+	3+	3+	-	-	-	-	-
-	3+	1+	3+	2+	-	-	3+	2+	1+	-	-	-	-	-
-	2+	-	3+	1+	-	-	3+	2+	2+	-	-	-	-	-
-	3+	3+	3+	3+	-	-	3+	3+	3+	-	-	-	-	-
-	3+	3+	3+	3+	-	-	3+	3+	3+	-	-	-	-	-
-	3+	3+	3+	3+	-	-	3+	3+	3+	-	-	-	-	-
-	-	-	3+	3+	-	2+	3+	-	-	-	1+	2+	-	2+
2+	-	-	-	-	3+	3+	-	-	-	-	3+	2+	-	2+
-	-	-	-	-	3+	3+	-	-	-	-	3+	3+	-	3+

/Tr indicates separate absorptions with organisms of opaque and transparent colonies respectively

45 °C With this illumination the opaque colonies is whitish colour and the transparent ones were whitish greyish on ocular inspection when the CMT plate is placed on the glass plate of the stereomicroscope and illuminated from below Strains were preserved as described previously (3)

Antisera

Antisera against the MOVP strains were obtained on rabbits as described previously (9) Antisera against C cells of T2 opaque and transparent colonies of the IC strains 670 SOA and P9 were obtained by immunizing rabbits with formalin fixed whole cells (3) so for each of the opaque and transparent colony morphology variants The first dose was 0.5 ml

1-5 ml of the antigen suspension was injected intramuscularly

Antisera were prepared as described previously (3)

Antisera were prepared as described previously (3) The supernatant of sonicated organisms (200 mg ml⁻¹ wet weight) (3) saline or lithium chloride extracts (see below) were used as antigens

Co agglutination

The production stabilization and coating of protein A-containing staphylococci with antibodies and the performance of the COA tests followed the procedures described previously (1-3) Whole GC cells heated at 100 °C for 30 or 60 min and in some experiments lithium chloride or saline extracts (see below) were used as test antigens Negative reactions were graded - weak 1+ moderately strong 2+ and strong or very strong reactions 3+

Antisera were prepared as described previously (3) The supernatant of sonicated organisms (200 mg ml⁻¹ wet weight) (3) saline or lithium chloride extracts (see below) were used as antigens

Preparation of Antigen Extracts

GC organisms were suspended in sterile 0.15 M saline at a concentration of 200 mg ml⁻¹ and boiled in water bath for 30 min as described for preparation of meningococcal group B serotype antigens (4) The

supernatant was collected after centrifugation at $3\,000 \times g$ for 20 min. Correspondingly the GC organisms were suspended in 0.2 M LiCl-0.1 M Na acetate pH 5.6 and treated for 2 h at 50 °C during vigorous stirring on a magnetic stirrer (5). The suspension was centrifuged at $3\,000 \times g$ for 20 min and the supernatant collected. Both this lithium chloride extract and the saline extract were used in some of the COA and C LIE & R LIE tests as described under Results.

Treatment with Pronase and Periodate

Whole cells heated at 100 °C were treated with pronase and periodate as described previously (10).

RESULTS

1) Co agglutination Reactions with Colour/Opacity Colonial Variants

Organisms of the MOMP and Micro IF reference strains and of colour/opacity variants of three isogenic GC strains were tested with COA reagents for antigen class W (10) and with reagent staphylococci coated with absorbed antibodies for the variants of the three isogenic strains. Pertinent COA results are shown in Table 1.

Strains 670 and SOA reacted with the reagents for antigen group W/I and strain P9 with W/II. The results were similar for the colour/opacity colonial variants of the isogenic strains. It can also be seen that strains 670 and SOA reacted with reagents corresponding to group A strains and P9 with reagents for group B strains of the Micro IF system of Wang *et al.* (14). Correspondingly

reagent staphylococci coated with anti 670 or SOA absorbed with P9 reacted with the MO strains of antigen group W/I and group A strains the Micro IF system and those of anti P9 with 670 or SOA with the MOMP strains antigen group W/II and with two of the group C strains.

Table 1 shows however that these reaction patterns were dependent on the colour/opacity colonial variants of the organisms used for immunization. Absorption and co agglutination of anti 670 Op absorbed with P9 Op or Tr reactions with the MOMP strains of antigen group W/I and with the group A strains of Micro IF expected but in addition also with strain S-11 group W/II. Anti 670 Tr absorbed with P9 Op or Tr reacted with group W/I strains but no reaction was detected with S-12. This strain also reacted with anti 670 Op absorbed with SOA Op or Tr even after absorption with 670 Tr. It can be seen that these reagents also reacted with organisms 670 Op colonies but not with those of Tr or any of the MOMP or Micro IF strains. The reaction was found to be sensitive to pronase in the same way as a corresponding colony morphology dependent reaction noted for SOA Op with anti Op absorbed with 670 Tr. This reagent reacted with MOMP strains of group W/II but also with strains B 2 and R 11 of W/I and with one group C and two group II strains of Micro IF. In addition, strain 4286 of group A. Anti P9 Tr did not react with B 2 R 11 or 4286 regardless of absorption but reacted with group W/II and group C strains.

TABLE 2 Demonstration by Crossed line or Rocket line Immunoelectrophoresis of Antigenic Differences in Preparations from Colour/Opacity Colonial Variants of Isogenic Strains

Anti GC antisera used in C LIE or R LIE	GC antigens in the intermediate agar gel	Number of specific precipitation lines formed in C LIE or R LIE with			
		670 T ₂ Op	670 T ₂ Tr	SOA T ₂ Op	P9 T ₂ Op
670 T ₂ Opaque	670 T ₂ Opaque	1	1	1	1
" "	" Transp	1	1	1	1
" "	SOA T ₂ Opaque	1	1	1	1
" "	" Transp	1	1	1	1
" "	P9 T ₂ Opaque	2	1	1	1
" "	" Transp	1	1	1	1
670 T ₂ Transp	670 T ₂ Opaque	1	1	1	1
" "	" Transp	1	1	1	1
P9 T ₂ Opaque	670 T ₂ Opaque	1	1	2	3
" "	" Transp	1	1	2	3

a) Fig. 11C refers to number of specific precipitation lines observed

TABLE 3 Coagglutination Tests of 58 Unselected Gonococcal Strains Distribution by Class IV Reagents and the Occurrence of Colony Morphology Dependent Reactions

Reagent staphylococci coated with absorbed anti GC antibodies	Tentative antigen class pattern	COA reaction pattern obtained with the 58 isolates										
Anti E 5 abs w N 10 D 4 n C 3 Y 15 n C 3	W/I	+	+	+	+			ca ¹				
		+	+			+			ga ¹			
N 10 n D 4 n S 12 A 1	W/II						ca ¹	+	+	+	+	ca ¹
								+	+	+		
n F 6 n A 1 & B 2	W/III											+
Number of reacting strains		19b ¹	4	1	1	1	14	1	1	1	12	3

c c colony morphology dependent reaction

1. Colony morphology dependent reactions (Op/Tr 3 + / +) were obtained for three strains with one of the reagents

On the whole immunization absorption and to

3) Distribution within Antigen Classes H and J of

1) Immunoelectrophoresis of Colour/Opacity Color
1a) Variants of Isomic Strains

CIE and RIE with antigen containing intermediate gel were performed with antigen antibody combinations of both the opaque and transparent variants of the isogenic strains and the heterologous strains. Examples of these tests are summarized in Table 2. It will be seen that the antigenic preparations of 670 Op had an extra antigen not present in 670 Tr which is in agreement with the COA results. Preparation of 670 and SOA shared cross reactive antigens also shown by the COA results (Table 1). The antigenic differences between P9 and 670 and SOA were confirmed in the IE tests.

The antigens responsible for the specific precipitation lines were present in both

1. Patterns of these were however somewhat different in CIE tests. Thus somatized organisms gave a two peak precipitin line as previously described (3) while saline and LiCl extracts gave one peak precipitin lines close to the antigen well. These extracts gave the same reactions as whole cells in the CoA test (Table 1).

unselected gonococcal strains from male and female patients were tested with COA reagents for amigen classes W and J. The W/II reagent prepared with anti-U14 absorbed with R11 (see Table 1) was excluded since it provided no further information except subdivision of W/II. The W/III reagent

u. lugens in organisms from colonies with opaque colony morphology. However, the previously described 1/6 reagent (10) prepared from anti F 6 absorbed with A 1 and B 2 did not show any cross reactivity with strains of antigen class W/11 and was thus included as a new W/11 reagent instead of that prepared from anti F 6 absorbed with B 2 and U 14. The overall results are shown in Tables 3 and 4.

All the 58 strains could be classified by class W reagents: 26 by W/I, 29 by W/II and three strains by W/III. Colony morphology dependent reactions were demonstrated for four (approx. 7%) of the strains, three of which had the combination W/I-II or W/II-III. Organisms from opaque colonies were responsible for these extra reactions. Only 25 of the strains (approx. 43%) were found to be positive for reagents Cc and were found

TABLE 4 Co-agglutination testing of 58 Unselected Gonococcal Strains Distribution by Class J Reagents and Occurrence of Colony Morphology Dependent Reactions

Reagent staphylococci coated with absorbed anti GC antibodies		Tentative antigen class pattern	COA reaction pattern with the 58 isolates									
Anti D-4	abs w B-2 & C-3	J/4	+	c ^a								
» U-14	» C-3	J/14	+	+	+	+						
» X-9	A-1 & B-2	J/9					+					
» S-12	» X-9	J/12			+					+	+	
Number of reacting strains			1	1	1	7	5	1	1	7	1	11

^a c = colony morphology dependent reaction

from opaque colonies were responsible for four of these reactions

Two or three GC isolates were obtained from different urogenital sites (urethra, cervix or rectum) in 14 patients, making 34 isolates in all. They were all tested by the class W/I and W/II and four class J reagents. Identical or similar COA patterns were obtained for all the 34 isolates by the class W reagents, and for all the 16 isolates reacting with the class J reagents. There were only two colony morphology dependent reactions with the class W and two with the class J reagents. One isolate obtained from a patient one week after the first three positive cultures (from urethra, cervix and rectum) gave identical results as those of the previous isolates.

The relatively low incidence of colony morphology dependent reactions observed with class W reagents with unselected strains was in striking contrast to the reactions obtained with class M reagents active against periodate sensitive antigens as described previously (10). A total of 14 of the 58 strains (24%) showed colony morphology dependent reactions. Ten strains (17%) gave reactions only with organisms from opaque colonies and 6 strains (10%) only with organisms from transparent colonies with at least one class M reagent. Two of these strains gave reactions only with organisms from opaque colonies with some reagents, and only with organisms from transparent colonies with other class M reagents.

When the paired isolates (34 strains) from the 14 patients were examined with the class M reagents and the colony morphology dependent reactions discussed above omitted, differences were noted for most reagents with strains from different sites of the same patient. Eight of the 14 patients had strains that differed in the same way with

organisms from both opaque and transparent colonies in the isolates from different sites of the same patient.

DISCUSSION

The previously described serological classification *Neisseria gonorrhoeae* by co-agglutination (10) was confirmed in the present study. We could also show that immunization of rabbits with organisms classified in this way gave antibodies which produced the expected results when used for preparation of COA reagents. We found, however, that the colour/opacity variants of isogenic T colonies, recently described by Swanson (11, 12) as opaque and transparent respectively were of importance when used for both immunization and for absorption and agglutination. Organisms from opaque T2 colonies gave extra antibodies compared to those from transparent colonies, were usually more efficient for absorption, and contained an extra pronase sensitive antigen factor(s). This was true for the antigen classes tentatively designated W and J.

These findings are of interest, since Walstead et al. (13) and Swanson (12) have shown that organisms from opaque T2 colonies of isogenic strains contain extra proteins as demonstrated by polyacrylamide gel electrophoresis of outer membrane preparations. They do not seem to be associated with pili, since they were absent in piliase transparent colonies of the isogenic strain Walsby et al. (13) and Swanson (12) also showed that they were not destroyed by heat at 100 °C. This is in agreement with our results, since the extra COA reactions of these organisms were demonstrated with heated (100 °C for 30-60 min) ...

It is noteworthy that the use of either transparent or opaque organisms was of minor importance in preparation of COA reagents for the tentatively defined antigen class W while it was of major importance in preparation of a reagent typical for antigen class J. We have shown previously that both reagents could be produced by absorption of antibodies with strains with a close antigenic relatedness (10). This was confirmed in the present work for example by absorption of anti 670 Opa III organisms of SOA which were of the same W antigen class as 670. However antigen class J could be demonstrated only with organisms from opaque colonies of the isogenic strains. These reactions were also found to be sensitive to pronase which we had previously shown was typical for some class J reactions.

Walstad *et al.* (13) and Swanson (11, 12) recently suggested that organisms of dark-coloured i.e. opaque colonies might have antigenic characteristics other than those of light-coloured transparent organisms of isogenic strains. In the present work we could demonstrate both by co-agglutination and immunoelectrophoresis that organisms from opaque T2 colonies had extra antigen(s) as compared to those of transparent colonies of isogenic strains. We have also found that the absorbing capacity of organisms of T3 or T4 colonies is lower than that of opaque or transparent T2 colonies (unpublished observations). Our findings might explain some of the antigenic variations demonstrated by Wilson

the feasibility of the co-agglutination method for serological classification of *Neisseria gonorrhoeae* reported in previous works (3, 9, 10) was confirmed in the present study. Thus all 58 GC isolates from patients could be classified by COA reagents for previously described antigen class W (10) while

genital and/or rectal sites in the same patient. This is in agreement with the findings reported in a previous work of repeated isolates of β -lactamase producing gonococci from the same patient (10). It is also of interest to note that the incidence of colony morphology dependent reactions was low with reagents for antigen class W but at least three to four times as high with available reagents for antigen class J when unselected GC strains were tested. It was also found that organisms from opaque colonies were responsible for almost all extra reactions. This was in striking contrast to the reagents for antigen class M (10). All strains were readable but tests with these reagents showed that, from both opaque and transparent

colonies of isogenic strains were responsible for variable reactions. The reasons for this are not yet clear, but they might be due to the cloning of colonies with different class M antigens rather than an effect of colony morphology. This was supported by the frequent finding of strains from different sites in the same patient with identical and stable class W but differing class M patterns. Further investigations are needed to elucidate these matters.

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" U-14	"	"	C-3	J/14		+	+	+				
" X-9	"	"	A-1 & B-2	J/9	c				+	"		
" S-12	"	"	X-9	J/12			+				+	+
Number of reacting strains					1	1	1	7	5	1	1	7

a) c = colony morphology dependent reaction

from opaque colonies were responsible for four of these reactions

Two or three GC isolates were obtained from different urogenital sites (urethra, cervix or rectum) in 14 patients, making 34 isolates in all. They were all tested by the class W/I and W/II and four class J reagents. Identical or similar COA patterns were obtained for all the 34 isolates by the class W reagents, and for all the 16 isolates reacting with the class J reagents. There were only two colony morphology dependent reactions with the class W and two with the class J reagents. One isolate obtained from a patient one week after the first three positive cultures (from urethra, cervix and rectum) gave identical results as those of the previous isolates.

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There was no disagreement with our results, since the extra CO reactions of these organisms were demonstrated with heated (100 °C for 30-60 min) whole cells.

IMMUNOPEROXIDASE AND ELECTRON MICROSCOPY STUDIES OF STAPHYLOCOCCAL LIPOTEICHOIC ACID

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Aasjord P & Grøv A Immunoperoxidase and electron microscopy studies of staphylococcal lipoteichoic acid Acta path microbiol scand Sect B 88 47-52 1980

Immunoperoxidase technique together with electron microscopy shows that lipoteichoic acid (LTA) of *Staphylococcus aureus* Cowan I is attached to the membrane and penetrates the whole cell wall. A diffuse zone outside and no peroxidase reaction product inside the wall when whole cells were treated with antibody prior to embedding may indicate (i) that LTA is exposed to reaction with antibodies outside the wall and (ii) that all or most of the anti LTA antibodies used are of the IgM class and thus unable to penetrate the wall. Thin sections of strain Wood 46 showed the same picture as Cowan I but treatment of whole cells before embedding gave no diffuse zone outside the wall. This may be due to a thicker wall as found by electron microscopy and/or shorter LTA-chains of strain Wood 46 than those present in the wall of Cowan I.

Key words: Staphylococcal lipoteichoic acid, immunoperoxidase, electron microscopy.

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In two previous papers (19, 20) staphylococcal cytoplasmic membrane and lipoteichoic acid (LTA) were isolated and examined chemically and serologically. LTA was found to constitute only a minor part of the isolated membrane. Considering the membrane as an attachment site for LTA, this structure has been referred to as membrane teichoic acid (2, 10, 13, 15). There are, however, observations indicating that LTA penetrates the cell wall and depending on the length of the glycerolphosphate backbone is exposed on the surface of the bacteria (4, 5, 18). The immunoperoxidase method and electron microscopy (1, 8) are utilized in the present study to localize LTA of *Staphylococcus aureus* strains Cowan I and Wood 46 in order to decide how LTA is related to the cell wall.

MATERIALS AND METHODS

Strains

Staphylococcus aureus strain Cowan I (NCTC 8530) was used for the preparation of protoplasts and LTA. (19) *Staphylococcus aureus* strain Wood 46 (NCTC 10344) being protein A negative was used as a control cell and source for polysaccharide A (poly A).

Growth of Bacteria

Blood agar, nutrient broth and protoplast medium were prepared as previously described (19).

Cultivation of *S. aureus* strains Cowan I and Wood 46 and isolation of protoplasts by incubating bacteria in sucrose medium to which was added 1000 IU of penicillin was carried out as earlier (19).

Antigen Preparations

Protein A, poly A and LTA were prepared as described in (7), (9) and (19) respectively.

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Growth of Bacteria

Blood agar, nutrient broth and protoplast medium were prepared as previously described (19).

Cultivation of *S. aureus* strains Cowan I and Wood 46 and isolation of protoplasts by incubating bacteria in sucrose medium to which was added 1000 IU of penicillin was carried out as earlier (19).

Antigen Preparations

Protein A, poly A and LTA were prepared as described in (7), (9) and (19) respectively.

Deceased June 26th 1979

Sera

Antisera to LTA were produced in New Zealand White rabbits of the Institute's breed by injecting a mixture of LTA and Freund's complete adjuvant (Difco, USA) into one of the rabbit's hind foot pads, followed by four injections given intramuscularly of a mixture of LTA and Freund's incomplete adjuvant (Difco, USA). Each dose contained 2 mg of antigen and was given in intervals of two weeks.

The anti-LTA serum used in the immunoperoxidase technique was absorbed on a Sepharose protein A column (14) to remove protein A reactive immunoglobulins. A peroxidase labelled anti-rabbit IgG (heavy and light chain) produced in swine (DAKO-Immunoglobulins, Copenhagen, Code P2190) was used for the immunoperoxidase studies.

Serum Tests

The peroxidase labelled serum and the anti-LTA serum were diluted two-fold and tested with bacterial cells by slide agglutination. Subagglutinating concentrations of the sera were used in the experiments.

Anti-LTA was tested for reaction with protein A and poly A by agar precipitation

Embedding Medium

Spurr (epoxy) medium was obtained from Taab Laboratories, England (16)

H₂O₂-Diaminobenzidine solutions (6, 8)

- 1) For treatment prior to embedding
75 mg 3,3'-diaminobenzidine-tetrahydrochloride (Fluka AG, Buchs Switzerland) in 100 ml 0.05 M Tris HCl buffer, pH 7.6, containing 0.01 per cent H_2O_2

Chemicals

Sodium cacodylate EM, and glutaraldehyde EM were purchased from Taab Laboratories

Sephacryl 4B for the preparation of the protein A column (14) was obtained from Pharmacia Fine Chemicals AB Uppsala Sweden

Peroxidase Labelling of Bacteria and Protoplasts Prior to Embedding (8)

Cells were incubated 1 h at room temperature in antiserum against LTA diluted 1:2 with TBS (0.05 M Tris buffered saline pH 7.6 containing 0.25 per cent MgSO_4 and 0.02 per cent sodium azide).

After washing four times with TBS peroxidase-conjugated swine-anti-rabbit IgG diluted 1:30 in TBS, was added to the cells and incubated at room temperature for 10 min, followed by washing four times with TBS. The cells were then fixed with 1 per cent glutaraldehyde in 0.1 M phosphate buffer, pH 7.2 for 20 min at 4 °C, followed by thorough washing (3 × 1 h) in phosphate buffer. The bacteria were transferred to the H₂O₂/diaminobenzidine solution 1, incubated for 10 min at room temperature and washed four times with the buffer.

The further treatment of the bacteria with OsO_4 dehydration was carried out using the method *Kellenberger et al* (11) Embedding took place in Spurr medium (16)

Protoplasts had to be fixed for 20 min in 0.1 M phosphate buffer, pH 7.2, containing 1 per cent glutaraldehyde, 1.6 M sucrose and 0.25 per cent MgSO_4 before further treatment. The subsequent steps took place in media without sucrose as for whole cells. Final samples were cut into thin sections of about 60 nm thickness (estimated by reflection of light) with ultramicrotome (LKB-Produkter, Sweden) and transferred to grids (150 mesh, formvar coated). The grids treated with 2 per cent aqueous uranyl acetate for 1 min, thoroughly rinsed in water, and then with 0.5 per cent lead citrate for 15 min and washed in water.

All electron microscopy was carried out on a Hitachi 12A transmission electron microscope (TEM) at 120 kV.

Peroxidase-Labeling of Thin Sections of Bacteria and Protozoa (8)

After fixation in 1 per cent glutaraldehyde in 0.1 cacodylate buffer, pH 7.4 (20 min, 4 °C), and wash with the buffer (3 × 1 h), the cells were dehydrated, embedded in the Spurr medium, cut into thin sections, and transferred to grids as described earlier. The Spurr medium of the thin sections was etched by floating grids on drops of fresh aqueous 10 per cent H_2O_2 for 1 min and then washed three times in beakers of deionized water. The grids were subsequently incubated on drops of anti-LTA serum and peroxidase-conjugated

the solution while stirring. Anti LTA serum and peroxidase-conjugated serum were diluted in TBS and 1:30 respectively.

Treatment with uranyl acetate and Reynolds's citrate prior to microscopy was performed as described earlier.

Except for the fixation performed in 0.1 M cacodylate buffer containing 1.6 M sucrose and 0.25 per cent MgSO_4 , protoplasts were treated like the nonbacteria.

RESULTS

The electron micrographs of protoplasts showed a dark narrow line covering the membrane of protoplasts were labelled prior to embedding (Fig. 1A) Control cells not treated with anti-LTA serum do not show this dark line (Fig. 1B)

When thin sections of *S. aureus* Cowan I, treated with anti-LTA serum and peroxidase-conjugated serum, peroxidase reaction product covered the whole of the cell wall in addition to the membrane (Fig. 2A). In the absence of anti-LTA serum, no reaction product occurred on the thin sections (Fig. 2B). The reaction product



Fig. 1. A Electron micrograph of a *S. aureus* Cowan I protoplast treated with anti LTA and peroxidase-conjugated rabbit IgG prior to embedding. Peroxidase reaction product is seen to be present on the protoplast membrane. $\times 30\,000$. B Protoplast from *S. aureus* Cowan I not treated with anti LTA serum. $\times 30\,000$.

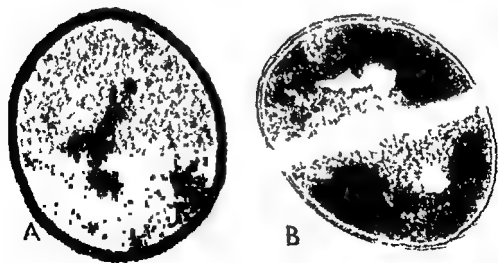


Fig. 2. A Electron micrograph of a thin section of a *S. aureus* Cowan I cell treated with anti LTA and peroxidase-conjugated rabbit IgG. Peroxidase reaction product seems to be present both throughout the cell wall and on the membrane. $\times 80\,000$. B Thin section of a *S. aureus* Cowan I cell not treated with anti LTA serum. $\times 80\,000$.

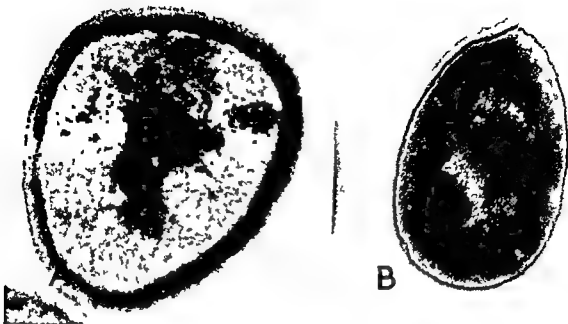


Fig 3 A Electron micrograph of a thin section of a *S. aureus* Wood 46 cell treated with anti LTA and peroxidase conjugated anti rabbit IgG. Peroxidase reaction product seems to be present both throughout the cell wall and membrane $\times 80\,000$ B Thin section of a *S. aureus* Wood 46 cell not treated with anti LTA serum $\times 80\,000$

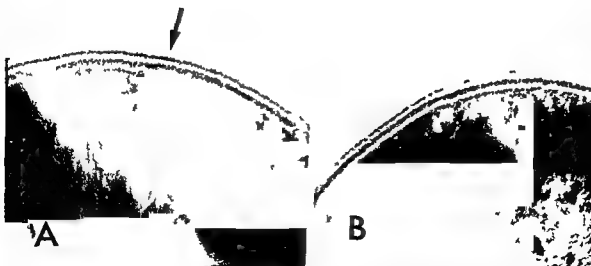


Fig 4 A Electron micrograph of a *S. aureus* Cowan I cell treated with anti LTA and peroxidase-conjugated rabbit IgG prior to embedding. Notice the dark zone of peroxidase reaction product outside the cell wall (arrows) which may be due to reaction with IgM immunoglobulins $\times 100\,000$ B *S. aureus* Cowan I not treated with LTA serum $\times 100\,000$

Wood 46) gave the same result (Fig. 3) indicating a reaction between the LTA of Cowan I and Wood 46. Cowan I cells treated with anti LTA serum and embedding showed a diffuse zone outside the cell wall (Fig. 4A) in contrast to the control without anti LTA (Fig. 4B) indicating that antibodies were bound to the bacterial surface. Wood 46 cells treated in the same way did not show any peroxidase reaction corresponding to an anti LTA binding.

DISCUSSION

The dark line which appeared outside the protoplasts after reaction with specific antibodies (anti LTA) and peroxidase labelled anti rabbit IgG (see Fig. 1A) confirms that LTA is attached to the membrane. Labelling of protoplasts on thin sections was not successful probably because LTA is easily washed from the membrane by washing during preparation of the samples.

The experiments with labelling of thin sections of *S. aureus* Cowan I showed further that LTA structurally is located both in the cytoplasmic membrane and in the cell wall (see Fig. 2A). Also in thin sections of strain Wood 46 the entire cell wall showed reaction (see Fig. 3A) demonstrating cross reaction between LTA of the two strains and a similar pattern of occurrence. The serum employed showed no protein A or polysaccharide A reactivity. No glutination of whole cells was observed (1:2 dilution of serum) thus indicating the absence of glutinins.

When whole Cowan I cells were treated with anti LTA serum prior to embedding there was no reaction on the cell wall or membrane but a diffuse zone appeared around the external surface of the cell (see Fig. 4A). This may be due to antibodies against LTA being of the IgM class (3, 17) and thus their size unable to penetrate the cell wall. Cells of the IgG class have previously been shown to penetrate the wall (8). The diffuse zone outside the wall of strain Cowan I thus indicates that part of the LTA reaches the surface of the cell. Strain Wood 46 failed to give such a diffuse zone. The LTA molecule is reported to have a variable number of subunits in bacteria giving rise to

That IgM antibodies are involved in the anti LTA activity is also indicated by a weaker reaction than normal for IgG antibodies (8). This may be because the peroxidase labelled antibodies against IgG only react with the light chains of IgM. Furthermore involvement of IgM antibodies in the anti LTA response is discussed in (21).

The presence of LTA in the wall of *S. aureus* strains and the different degree of penetration are observations which must be considered in studies on cell wall antigens.

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The micrographs indicate that the Wood 46 cell wall is thicker than that of Cowan I (see Fig. 2A) and this may be the reason why Wood 46 cells

immunoglobulins (8-14) was applied to a Sephadex G 200 column (3 × 45 cm) and eluted with phosphate buffered saline (PBS) pH 7.2 containing 0.02 per cent sodium azide. The eluate was collected in 2 ml fractions at a flow rate of 6 ml per h. Fractions assumed to contain the same immunoglobulins were pooled, concentrated to a volume of 2 ml in collodion bags (Sartorius Membran filter, Göttingen) and tested for reaction with LTA using countercurrent immunoelectrophoresis (I).

To identify the immunoglobulin classes the pooled fractions were tested with goat anti rabbit IgG (γ specific) and sheep anti rabbit IgM (μ specific) by double diffusion in agar gel. Fractions were also used in the immunoperoxidase experiments.

Countercurrent Immunoelectrophoresis (I) of the Anti LTA Fractions

The electrophoresis was carried out for 1 h on a 10 × 10 cm glass plate coated with 1 per cent agarose in 0.05 M veronal buffer pH 8.6 at 10 Volt per cm.

Peroxidase Labelling of Thin Sections of Bacteria (5)

The bacteria *S. aureus* strains Cowan I and Wood 46 were fixed with glutaraldehyde, dehydrated, embedded in the Spurr epoxy medium and cut into thin sections with an ultramicrotome (LKB Produkter, Sweden) (see 14).

The labelling took place by incubation of H_2O_2 etched thin sections with the pooled anti LTA fractions followed by incubation with peroxidase conjugated anti rabbit IgG and reaction with the diaminobenzidine solution. The procedure was as described previously (5, 14).

Electron microscopy was carried out on a Hitachi HU 12A transmission electron microscope (TEM) at 100 kV.

RESULTS

Two main immunoglobulin peaks and a small intermediate one appeared on fractionation of the rabbit anti LTA serum on the Sephadex G 200 column (Fig. 1 I). Countercurrent immunoelectrophoresis showed that the fractions A and II as well as unfractionated anti LTA serum precipitated with LTA (Fig. 1 II) whereas fractions C and D did not. The protein A reactive immunoglobulins bound to the Sepharose protein A column gave on testing no precipitation line with LTA.

Double diffusion in agar showed that the fractions A and B contained the IgM class of immunoglobulins only and D the IgG class (Fig. 1 III). Fraction C contained IgG and most probably also IgA but in too low concentrations to be observed in double diffusion. Fraction B from the IgM peak and the fractions C and D were further used in the immunoperoxidase experiments.

Labelling thin sections of bacteria with the separated fractions showed that only those sections

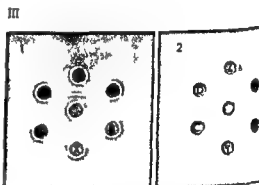
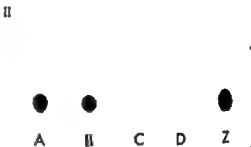
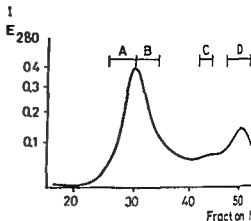


Fig. 1 I Fractionation of anti LTA serum on Sephadex G 200. A and B mark the IgM peak, D the IgG peak. Fractions from region C contained IgG and possibly IgA. Fractions were collected as indicated by bars.

II Countercurrent immunoelectrophoresis of the LTA fractions in 1 per cent agarose for 1 h in 0.05 M veronal buffer pH 8.6, 10 Volt per cm, then stained with Coomassie Brilliant Blue R (Sigma, USA). Upper wells contain LTA (1 mg/ml) and the lower wells contain the serum fractions (A, B, C, D) and unfractionated LTA serum (Z).

III Fractions A, B, C, and D tested against goat anti rabbit IgG (γ specific) (1) and sheep anti rabbit IgM (μ specific) (2) in agar precipitation. Glass plates coated with 1.2 per cent water agar were used. The test sera

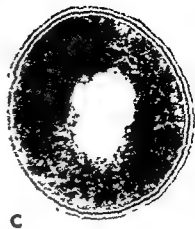
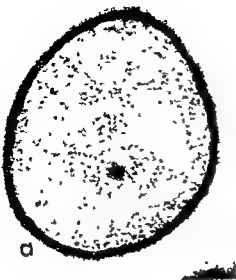


Fig. 2. Electron micrographs of immunoperoxidase treated thin sections of *S. aureus* strain Cowan 1 (a) and strain Wood 46 (b) treated with the IgM fraction from Sephadex G 200. Peroxidase reaction product is seen on the cell wall of both bacteria. Treatment of thin sections of Cowan 1 cells with the IgG fraction (c) gave no peroxidase reaction on the cell wall. Magnification 80 000 \times .

DISCUSSION

Fractionation of the rabbit anti LTA serum followed by countercurrent immunoelectrophoresis precipitation in agar and immunoperoxidase technique applied to bacterial thin sections clearly showed that IgM is the immunoglobulin which interacts with LTA. The anti LTA serum was depleted of protein A reactive immunoglobulins and did not react with the wall teichoic acid (poly A).

The IgM peak was collected in two separate parts (A and B). This was done to avoid a possible interference of other serum proteins or complexes which may be present in fraction A. However, fractions A and B gave identical results in the experiments.

The results are in accordance with other observations that the specific IgM antibodies are unable to penetrate the cell wall in contrast to IgG (5). Staining is relatively weak due to peroxidase labelled anti rabbit IgG (heavy and light chain). Thus in the present experiments only reactions with light chains of the IgM antibodies occurs.

Anti LTA antibodies of the IgM class are previously reported by Wicken & Knox (11).

which were treated with the fraction containing IgM did show the presence of peroxidase reaction product (Fig. 2a and b). Treatment of thin sections with the fractions C and D containing IgG (and probably IgA as well in C) showed no reaction. Nor was any reaction observed by treatment of thin sections with the peroxidase-conjugated serum alone.

All four anti LTA sera used in the experiments

especially antibodies directed against sugar moieties of the LTA. Immunoglobulins of the IgG class are reported to be directed against the glycerophosphate backbone (11). In the present experiments no anti-LTA activity was observed in the IgG fraction. Gas liquid chromatography showed that the extraction procedure used to isolate LTA from *S. aureus* Cowan I leaves the molecule intact (12). Thus, if antibodies against the glycerophosphate backbone are of the IgG class, no antibodies of this specificity are present. The degree of sugar substitution has been shown to influence the antibody response to the polyol phosphate backbone (7). Furthermore, the animal species, the vaccine composition and the route of administration selected are important factors in this connection.

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BRIEF REPORT

LACK OF EVIDENCE FOR AN ASSOCIATION BETWEEN INFECTION WITH *CHLAMYDIA TRACHOMATIS* AND CROHN'S DISEASE, AS INDICATED BY MICRO-IMMUNOFLOURESCENCE ANTIBODY TESTS

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Mårdh P, A, Ursing B & Sandgren E. Lack of evidence for an association between infection with *Chlamydia trachomatis* and Crohn's disease, as indicated by micro-immunofluorescence antibody tests. Acta path. microbiol. scand. Sect. B 88: 57-59, 1980.

A significant difference has recently been reported in the occurrence of serum antibodies in the lymphogranuloma venereum (LGV) immunotypes of *Chlamydia trachomatis* in patients with Crohn's disease and controls. In the present study sera from 107 patients with Crohn's disease, 33 males and 74 females, were tested for antibodies to *C. trachomatis* by an indirect immunofluorescence test, using two pools of antigens. These pools contained antigens to immunotypes TRIC D K and LGV 1-3 respectively. None of the patients had IgM antibodies to the organism. IgG antibodies occurred significantly more often in the female than in male patients. No correlation could be demonstrated between activity and duration of Crohn's disease and the results of the antibody tests. For comparison the sera of 50 puerperal women were studied. No difference in the occurrence of IgG antibodies was found between patients and controls, neither when using the two pools of antigen, thus indicating that the antibodies detected had probably been induced by TRIC rather than LGV organisms. To conclude the study did not indicate an association between Crohn's disease and infection with *C. trachomatis*.

Key words: Crohn's disease, *Chlamydia trachomatis*, serum antibodies, LGV, TRIC.

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The aetiology of Crohn's disease, the frequency of which seems to be increasing (10), is unknown. A variety of organisms, including anaerobic bacteria, mycobacteria and viruses, have been considered as possible aetiological agents (12). Recently Schuller and coworkers (5) in the US reported that they found antibodies to *Chlamydia trachomatis* of the lymphogranuloma venereum (LGV) prototypes in 38 of 55 patients with Crohn's disease, but significantly less often in patients with other gastrointestinal disorders and in healthy blood donors. Freis test has been reported positive in patients with Crohn's disease (8).

In calves *Chlamydia psittaci* may cause epithelioid granulomas (1). All the possible occurrences of gastrointestinal infections with *C. trachomatis* viz. the other exclusively human species of *Chlamydia* has so far been little explored.

One hundred and seven patients with Crohn's disease, 33 males and 74 females, were studied. They were selected at random among the approximately 300 patients with this disease at present under treatment at the Department of Infectious Diseases, University Hospital, Lund, Sweden. The diagnostic criteria for Crohn's disease used were the same as those presented elsewhere (3). Differentiation of the clinical material into active and non-active cases was based on a combination of clinical and laboratory parameters. Although considerable difficulties are encountered in attempting to determine the duration of disease in a given case, the material was divided into cases where there had been symptoms suggestive of Crohn's disease for three years or longer and those with symptoms of shorter duration. Fifty puerperal women from the same hospital catchment region served as controls.

Sera from the patients were tested for IgM and IgG

TABLE IgG Antibodies to *Chlamydia trachomatis* in Patients with Crohn's Disease in Relation to Sex of Patient, Duration and Activity of Disease and in Puerperal Control Women

Group	Sex	Duration (yrs) and activity of disease	No of patients studied	Titre of IgG antibodies to <i>C. trachomatis</i>					
				<16	32	64	128	256	>256
Patients	males	active	0	0	0	0	0	0	0
		<3							
		inactive	6	5	1	0	0	0	0
		active	6	6	0	0	0	0	0
		>3							
		inactive	21	19	2	0	0	0	0
	females	active	4	2	0	0	2	0	0
		<3							
		inactive	16	12	3	1	0	0	0
		active	10	8	2	0	0	0	0
		>3							
		inactive	44	31	4	5	3	1	0
Controls	females	-	50	38	6	1	0	2	0

antibodies to *C. trachomatis* by a micro immunofluorescence test (9) using two pools of antigen. One pool consisted of antigens of *C. trachomatis* immunotypes TRIC (trachoma inclusion-conjunctivitis agents) D K and the other of antigens of immunotypes LGV 1-3.

The occurrence of IgG antibodies to *C. trachomatis* reacting with LGV 1-3 is shown in the Table. None of the patients had such IgM antibodies. IgG antibodies to *C. trachomatis* occurred significantly more often in the female than in the male patients. No relationship was found between the presence of antibodies and the duration and activity of Crohn's disease. Of the controls 16% had IgG antibodies to *C. trachomatis* with a titre of >64 while the corresponding figure in the female patients was 12%.

With a few exceptions testing the sera by using the

to *C. trachomatis* was associated with acute stage Crohn's disease. Neither did the lack of IgM antibody to this organism suggest acute infections with *trachomatis* as being associated with that disease.

The finding that antibodies to *C. trachomatis* demonstrated significantly more often in female than in male patients with Crohn's disease is in conformity with studies on patients with no evidence of that disease. The similarity of the result in the tests using the different pools of antigens suggests that the antibodies demonstrated in the sera of the patients and controls probably been produced against TRIC organisms. Antibodies to these organisms cross react broadly with LGV antigens (11).

The study was supported by grant 16X 04509 from Swedish Medical Research Council.

among the sera containing antibodies to *C. trachomatis* and never exceeded one dilution step.

Our findings of antibodies to *C. trachomatis* in patients with Crohn's disease are not in principle in accordance with those of Schuller *et al.* (5) but are similar to those of two recent reports from the U.K. (6). The material consisted of 100 patients with Crohn's disease from the point of view of clinical parameters did not indicate that the occurrence of serum antibodies

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BRIEF REPORT

GROWTH INHIBITION OF *STREPTOCOCCUS MUTANS* STRAIN OMZ 176 BY XYLITOL

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Assev S Vægarud G & Rølla G Growth inhibition of *Streptococcus mutans* strain OMZ 176 by xylitol
Acta path microbiol scand Sect. B 88 61-63 1980

The present study showed that xylitol exhibited a dose related inhibition of the growth of *Streptococcus mutans* in a Brain Heart Infusion medium. It is suggested that the mechanism involved may be an effect of the translocation of glucose across the bacterial cell membrane. Sorbitol showed no similar effect but caused a delay in the reaching of the stationary phase.

Key words: Xylitol, sucrose substitutes, dental caries.

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Xylitol and sorbitol are used as sucrose substitutes in oral sweets to improve dental health because of the togenicity of sucrose particularly when consumed between meals. There are controversial reports in the literature concerning the effect of xylitol on the growth of *Streptococcus mutans* which is thought to be the primary aetiological agent of dental caries. *Makinen* (1972) showed that the growth of strain logbrit was inhibited by xylitol but reported normal growth of this strain 4-5 months after the continuous presence of xylitol in the diet. Growth inhibition was also reported by *avenger et al* (1978) whereas *Muhlemann et al* (1977) were unable to show any such effect in strain OMZ 176. The present study was initiated to compare the effect on the growth of *Streptococcus mutans* strain OMZ 176 of the addition of xylitol or sorbitol to the culture media.

Growth inhibition experiments 5 ml of a stationary phase culture was inoculated into 100 ml batches of sterilized (20 min 110 °C) BHI medium. The carbohydrate solutions were sterilized separately and added to the medium in order to obtain the concentration specified below. The cultures were then incubated at 37 °C.

1 ml aliquots were drawn at zero time and at every hour during the log phase and every second hour during the stationary phase. The last aliquots were taken 26 hours after inoculation. *Streptococcus mutans* strain OMZ 176 was grown in BHI with either no sugar added, with 2.5% glucose added or with 1%, 4% or 5% sorbitol or xylitol added.

Each series of experiments was carried out simultaneously with medium from the same batch and carbohydrates from the same solutions.

Results

The first series of experiments showed that 5% of xylitol in the medium inhibited growth compared with the basic BHI medium with no extra carbon source added. Addition of both xylitol and glucose to the cultures gave a reduction in growth as compared to addition of glucose alone and the inhibition was dose related (Fig. 1).

Similar experiments with sorbitol did not show such clear effects. Addition of 5% sorbitol to the basic medium gave a slightly higher growth after 24 hours although the stationary phase of growth was reached

Material and Methods

Bacteria *Streptococcus mutans* strain OMZ 176 was obtained from Dr. B. Guggenheim, Zurich.

Growth medium Bacto Brain Heart Infusion (BHI) containing 0.16% glucose was used as a basic medium.

Carbohydrates D-Sorbitol and Xylitol were purchased from Fluka AB and D (+) Glucose (anhydrous) from Merck.

Growth measurement The growth of the cultures was measured at 650 nm against water in a Zeiss PMQ II spectrophotometer.

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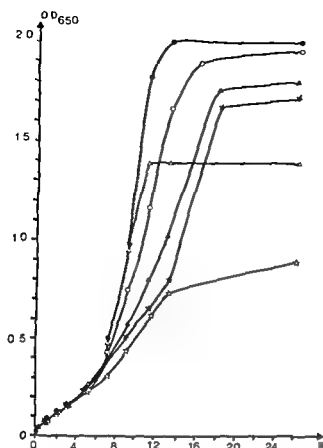


Fig 1 *Strep mutans* strain OMZ 176 grown in BHI with glucose and xylitol Δ — Δ no carbohydrates \bullet — \bullet 2.5% glucose \circ — \circ 2.5% glucose and 1% xylitol \blacktriangle — \blacktriangle 2.5% glucose and 4% xylitol \star — \star 2.5% glucose and 5% xylitol

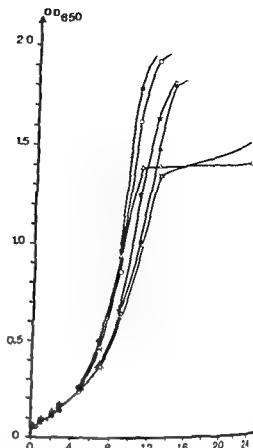


Fig 2 *Strep mutans* strain OMZ 176 grown in BHI with glucose and sorbitol Δ — Δ no carbohydrates \bullet — \bullet 2.5% glucose \circ — \circ 2.5% glucose and 1% sorbitol \blacktriangle — \blacktriangle 2.5% glucose and 4% sorbitol \star — \star 2.5% glucose and 5% sorbitol

later in the sorbitol culture. Addition of 1% sorbitol to a culture containing 2.5% glucose showed no effect on the final growth level. The addition of 4% and 5% of sorbitol had an inhibitory effect though this was less marked than with xylitol (Fig 2).

Discussion

Several reports have indicated that *Strep mutans* is unable to metabolize xylitol whereas sorbitol is metabolized slowly by this microorganism as measured by acid production in cultures (Havenaar *et al* 1978). Our experiments supported the observations of Makinen (1972) and Havenaar *et al* (1978) in demonstrating a reduction by xylitol on the growth of different strains of *Strep mutans*. This was particularly apparent when no other carbon source was added but could also be observed with glucose and the effect was dose related.

Sorbitol did not show any similar effect in the experiment where no glucose was added. However the stationary phase was reached later but the level of growth was higher after 24 hours in the sorbitol cultures thus indicating some metabolization of this carbon source by *Strep mutans*. Addition of sorbitol to cultures

containing extra glucose showed some reduced growth and a longer time used in reaching the stationary phase but this was not as marked as in experiments with xylitol.

The mechanism of the inhibition described is unknown. It seems conceivable that an effect on translocation of glucose across the bacterial cell membrane occurs either on the phosphotransferase system or through blocking of sugar-binding proteins (Makinen 1978). Xylitol is known to be a potent inhibitor of the D-Xylose isomerase of *Lactobacillus brevis* (Yamanaka 1969). It is not known whether or not the growth reducing effect of the substitutes may be of clinical significance in their use in the oral cavity but this seems possible.

Recent research has shown that dental plaque formed in the presence of xylitol exhibits changed properties as compared to plaque formed in the presence of sucrose; the former being less adhesive and less hydrated. It also contains less glucan and lipoteichoic acids (Rolla *et al* 1980). Growth inhibition of *mutans* by xylitol could conceivably be of significance in this respect in reducing the metabolic processes occurring in dental plaque. No similar data are available for

BRIEF REPORT

ENTEROTOXIN PRODUCTION BY *YERSINIA ENTEROCOLITICA* AND *YERSINIA ENTEROCOLITICA* LIKE MICROBES AT 22 °C and 37 °C

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Kapperud G Berdal B P & Omland T Enterotoxin production by *Yersinia enterocolitica* and *Yersinia enterocolitica* like microbes at 22 °C and 37 °C Acta path microbiol scand Sect. B 88 65-67 1980

A total of 32 strains of *Yersinia enterocolitica* and *Y. enterocolitica* like microbes were examined for their ability to produce *E. coli* ST like enterotoxin when grown at 22 °C and 37 °C using the infant mouse assay Enterotoxin production was indicated for 19 (59%) of the 32 strains examined Nine of ten sucrose non fermenting strains belonging to O serogroup 28 produced enterotoxin at both 22 °C and at 37 °C Eight of these strains were obtained from the intestinal contents of apparently healthy small mammals or from water samples at three different localities in Norway One O serogroup 28 culture was a reference strain received from Institut Pasteur Paris With the exception of O serogroup 28 enterotoxins were detected only after incubation at 22 °C The infant mouse assay was positive for all the four clinical isolates belonging to O serogroup 3/biotype 4 Six of 13 different reference strains were positive in this assay

Key words *Yersinia enterocolitica* enterotoxin

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In previous studies we demonstrated that *Yersinia enterocolitica* and *Y. enterocolitica* like microbes are ubiquitous in both terrestrial and freshwater ecosystems Scandinavia (2 3 4 5) A broad diversity of ecologically and biochemically different types were isolated However the significance of most of these strains as a cause of animal or human disease is unknown The epidemiology/epizootology of this kind yersiniosis is still not completely understood In recent years interest has been focussed on the search for pathogenicity factors among strains of *Y. enterocolitica* (7 8 9 11) This may represent an important clue towards a more complete understanding of the epidemiology and ecology of this microbial group i et al (8) reported that production of *E. coli* ST like enterotoxin was observed in strains of *Y. enterocolitica* and *Y. enterocolitica* like microbes

In the present investigation a pilot survey comprising *Y. enterocolitica* reference strains and clinical isolates indicated enterotoxin production at 37 °C by one representative of O serogroup 28 This observation prompted us to consider a more extensive material of this O serogroup

Materials and Methods

1 Bacterial Strains

A total of 32 cultures of *Y. enterocolitica* and *Y. enterocolitica* like microbes were examined Their serological properties and source of isolation are presented in Table 1 The strains fall into three different groups

1 Reference strains A total of 13 type strains representing different O serogroups of *Y. enterocolitica* were included Twelve strains were received from Institut Pasteur Paris one was obtained from Dr Winkblad Malmö

2 Clinical isolates Ten clinical isolates four of which belonged to O serogroup 3/biotype 4 (Wauters biotype scheme) were examined Nine strains were isolated from

Enterotoxin Assay

Enterotoxin activity was tested using the infant mouse assay (1). Sterile culture filtrate (0.1 ml) was injected through the abdominal wall into the milk filled stomach of three 3-4 day-old mice (NMRI/BOV/kp). Injected mice were kept at room temperature for four hours. The animals were killed and the entire intestine was removed to determine the ratio weight of intestine to remaining body weight. Ratios of ≥ 0.083 were considered positive. This value is accepted as a minimum for demonstration of *E. coli* ST enterotoxin in the infant mouse assay (10).

Results

Results are presented in Table 1.

The presence of *E. coli* ST like enterotoxin in culture was indicated for 19 (59%) of the 32 strains of *Y. enterocolitica* and *Y. enterocolitica* like microorganisms examined by infant mouse assay. Encompassed by this study are representatives of 16 O-serogroups, 11 of which showed enterotoxin production. Nine of ten isolates belonging to O-serogroup 28 (sucrose non fermenting) produced enterotoxin when cultured at both 22 °C and at 37 °C. With this exception, enterotoxin was demonstrable only when organisms were grown at 22 °C.

All the four strains belonging to O-serogroup 3/type 4 were positive for enterotoxin. Another six strains isolated from human patients with gastrointestinal disease belonging to other O-serogroups gave positive results.

Among the 13 reference strains examined, enterotoxin was detected for strains representing six different O-serogroups.

Discussion

All hitherto published investigations concerning enterotoxin production by *Yersinia enterocolitica* have not included O-serogroup 28. This O-serogroup was first isolated nine years ago from untreated drinking water in

Norway by Lassen (6). Reports of isolation from other countries are very sparse.

So far no pathogenic properties have been ascribed to O-serogroup 28. However, this serogroup acquires a new significance from the present study where it gave the first known indication of enterotoxin production by *Y. enterocolitica* at 37 °C. With the exception of this serogroup, enterotoxin production in *Y. enterocolitica* has been found only when strains were grown at temperatures lower than those prevailing in the bodies of mammals and birds.

For the present one should question the clinical significance of *Y. enterocolitica* produced enterotoxin. Despite this, in view of the present findings, the possibility of a pathogenic role of O-serogroup 28 should be reconsidered.

We want to thank Jørgen Lassen and Jorunn Sundar for the clinical isolates provided. The excellent technical assistance of Merete Mathiesen is gratefully acknowledged.

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human patients with gastrointestinal disease at the National Institute of Public Health, Oslo. One strain was isolated from swine.

3 Environmental strains Included are nine strains obtained from terrestrial and freshwater ecosystems in three different communities in Norway. Isolations were made from the intestinal contents of apparently healthy small mammals and from water samples (3). All strains were antigenically related to O serogroup III and did not ferment sucrose. All except one strain were Voges-Proskauer negative (both at 37 °C and at room temperature) and aesculin negative. Four strains were salicinate negative.

The procedure followed in serological and biochemical

characterization has been specified elsewhere. Strains were stored as stab cultures.

II Preparation of Sterile Culture Filtrates

The strains were inoculated into 5 ml broth (TSB) containing 0.6% yeast extract. Broths were incubated for 48 hours on a rotary shaker (200 rev/min) at room temperature (approximately 22 °C) and at 37 °C. The cultures were centrifuged (4000 rev/min for 30 minutes). Supernatants were filtered sterilely through filters (pore size 0.45 µm). Filtrates were stored and examined for the presence of enterotoxins within one week.

TABLE 1 Enterotoxin Production by Clinical and Non Clinical Isolates of *Yersinia enterocolitica* Like Microbes Indicated by Infant Mouse Assay

Strain no		Source	O serogroup	Enterotoxin produced 22 °C	3
96 IP	Reference strains	Chinchilla	4-32	-	
1476 IP	"	Water	4-33	-	
123 IP	"	Cow	5A	+	
885 IP	"	"	5-27	+	
102 IP	"	Human	6-30	-	
FY 50	"	"	8-19	+	
201 IP	"	Human	9	-	
867 IP	"	Human	16-29 Rh + ^a	-	
846 IP	"	Human	18	-	
845 IP	"	Human	20	+	
1367 IP	"	Human	22	+	
1474 IP	"	Water	28	S- ^b	+
1501 IP	"	"	34	-	
9595/76	Clinical isolates	Human	3		+
306/77	"	Human	1		+
365/77	"	Human	3		+
311/77	"	Swine	3		+
6526/76	"	Human	4-32		-
5367/76	"	Human	5		-
5831/76	"	Human	6-30		-
R37/77	"	Human	14		-
9728/76	"	Human	16	Rh +	-
R6/77	"	Human	NT ^c		-
11051 A	Environmental strains	Shrew	28	S-	+
9021 II	"	Shrew	28	S	+
9019 A	"	Shrew	28	S-	+
1003 A	"	Shrew	28	S-	+
10016 B	"	Shrew	28	S-	+
9008	"	Small rodent	28	S-	+
1030	"	Small rodent	28	S-	+
G2	"	Water	28	S-	+
G13 A	"	Water	28	S-	+

^a Rhamnose positive

^b Sucrose negative

^c Not typeable

A VACUUM GADGET FOR SAFE AND RAPID MOUNTING OF COVER SLIPS ON SLIDES WITH LIVING PATHOGENIC MICROORGANISMS

ERAST RASMUSSEN

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Rasmussen E A vacuum gadget for safe and rapid mounting of cover slips on slides with living pathogenic microorganisms *Acta path microbiol scand Sect B* 88 69-70 1980

A simple gadget is described for the safe and rapid mounting of cover slips on slides with infectious materials The instrument has worked satisfactorily in the mounting of more than 50 000 slides

Key words Safe cover slips pathogenic microorganisms

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Mounting of cover slips on slides for microscopy involves a certain risk when working with infectious materials Furthermore it is timeconsuming to pick up the cover slips and mount them carefully on large numbers of slides No instructions for facilitating this process were to be found in textbooks on microbiology and microscopy (1 2 4) To my knowledge of the writer no suitable and simple instrument is commercially available and it has not been confirmed that no other instrument is available

In Sweden the United Kingdom or the USA (3) A safe highly efficient and inexpensive vacuum gadget is presented here

CONSTRUCTION AND FUNCTION

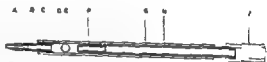


Fig 1 Construction of vacuum gadget for mounting cover slips For explanation see text

mm bore 7 mm A tube of stainless steel H length 100 mm is pushed 80 mm into the other end of G F is a tapered tube of plastic length 15 mm fitted to H The tip of F is a little uneven A steel ball 6 mm diameter is placed into D the partition between C and F F is connected to a vacuum pump by I a flexible tube of 2 mm bore

It is brought to I where it does not close completely This saves unnecessary suction of air and prevents noise from suction If the tip of A is blocked by a cover slip the compartment between the tips of A and F is evacuated and E is released

In operation the gadget is held like a pencil with the tip somewhat downwards A cover slip is taken by pressing the gadget gently on the cover slip perpendicular to its surface (Fig 2a) After less than one second the cover slip can be lifted (Fig 2b) The cover slip is mounted on the slide by pressing an edge of the

QUANTIFICATION OF BACTERIA IN OPERATION WOUNDS - AN EXPERIMENTAL INVESTIGATION WITH SWAB SAMPLING IN PIGS

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Nyström P-O Quantification of bacteria in operation wounds - an experimental investigation with swab sampling in pigs Acta path microbiol scand Sect B 88 71-77 1980

Quantitative recovery by swab cultures of *Staphylococcus aureus* or *Escherichia coli* from experimental wounds in pigs was studied for assessment of wound contamination in elective surgery. The recovery rate declined rapidly over the first hour. Ten minutes after seeding the wounds with known numbers of bacteria the recovery was respectively 0.7% and 4.1% for *S. aureus* and *E. coli*. Recovery was not affected by the size of the wound. Squeezing the swab onto the culture plate or rinsing the swab in saline and plating out the rinsing fluid immediately after sampling resulted in a three to four times higher yield than by using Stuart's transport medium for later plating. The results provide an indication of the discrepancy between the number of micro-organisms recovered from wounds in elective surgery and the number of micro-organisms required to produce suppurative infection in experiments on animals and in man.

Key words: Operation wounds, bacteria, quantification, pigs.

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The risk of post-operative wound infection is related to intra-operative contamination of the incutaneous wound with bacteria, as shown by the gher infection rate in patients with a positive suture from the wound on closure (Dillon *et al* 1969, Davidson *et al* 1971, Brote 1976).

Quantification of the bacterial population in the wound should provide information for further analysis of the role of bacteria in the pathogenesis of post-operative infections. However, little is known concerning the accuracy and significance of quantitative wound cultures in elective surgery, though experience is accumulating in the field (Taylor 1961, Burke 1963, Lilly *et al* 1970, Raachov 1974, 1976, 1979, Sanderson & Bentley 1976, Bartlett *et al* 1978, Scheibel *et al* 1978).

Obviously, counts of bacteria from wound cultures do not represent the true number of bacteria reaching the wound in the course of

surgery. It is unlikely that all bacteria can be removed from the wound by the sampling device or can be totally transferred to the culture medium.

The aim of this study was to give an experimental account of the relationship between the wound population of bacteria and the number of bacteria recovered from the wound by quantitative wound swab cultures. Attempts were made to identify some of the factors which influence the recovery rate.

MATERIALS AND METHODS

Pigs of the Swedish land race weighing about 20 kg were anaesthetized. Tracheotomy with endotracheal intubation was performed and the normal body temperature maintained. Physiological saline was infused intravenously during the experiments.

The sides of the animals were shaved and the skin disinfected with 0.5% chlorhexidine in 70% alcohol.

a



b



Fig 2 Vacuum gadget in operation picking up (a) transferring (b) and mounting (c) cover slip. For details see text.

slip gently on to the slide (Fig. 2c). It is released from the gadget as soon as the slide is not perpendicular to the gadget.

RESULTS AND DISCUSSION

The gadget has been used by laboratory technicians for mounting more than 50 000 cover slips with live pathogenic bacteria. The mount can be performed gently and rapidly. There is no risk of contamination of the operator, nor any of the fingers being cut.

The gadget can be used after a brief instruction. It is durable and can be produced at a low price.

Borge B. Jørgensen and J. Rius Nielsen are thanked for help with preparation of the manuscript.

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The results are shown in Tables 1 and 2. Mean recovery was 0.7% with *S. aureus* and 4.1% with *E. coli*. The differences between lowest and highest recovery from single wounds within each experiment (ten wounds same seeding) varied from 3 fold to 30 fold. When wounds were seeded with 1.2×10^5 CFU of *S. aureus* four out of ten wounds were culture negative and no culture showed more than two CFU.

Recovery followed an approximately linear relationship with inoculum thus suggesting that wound culture count was dependent mainly on the wound bacterial population up to 10^5 CFU per wound. A linear regression analysis of $y = \log$ wound culture count on $x = \log$ inoculum count was performed on the experiments with *S. aureus* in order to assess prediction errors involved. Roughly an allowance of one logarithm in either direction seemed to be involved when predicting wound culture count from inoculum count and aiming at 95% confidence in prediction. Inversely when predicting wound population from wound culture count, the responding margin of error was about one and half logarithms. However, available data did not

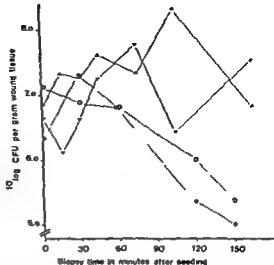


Fig. 2. Change in wound tissue concentration (CFU/g) with time after seeding experimental wounds in pigs with either *Escherichia coli* (solid line) or *Staphylococcus aureus* (broken line). A decline in tissue concentration of *S. aureus* was observed but not of *E. coli*.

yield good estimates of the variance components involved and in consequence the limits must be taken with caution.

Effect of Time on Recovery

To determine the recovery as a function of time after inoculation forty wounds in sets of ten were seeded with 1.2×10^5 *S. aureus* and sampled at 2, 10, 30 and 60 min respectively.

A non linear decline in recovery was observed (Fig. 1). At 2 min mean recovery was 4.4×10^3 CFU (3.6%) and this declined to 8.2×10^1 CFU (0.07%) at 60 min.

Corresponding results were obtained when wounds were seeded with *E. coli*. Mean recovery was 4.1% at 10 min and this declined to 1.8% when sampling was performed 30 min after seeding (Table 2).

The significance of time on recovery was evaluated further by performing the following experiment. Stab wounds 1 cm in length and 1 cm in depth were made and seeded by cotton swabs dipped in 10^8 CFU per ml of either *S. aureus* or *E. coli* which were allowed to remain in the wounds for 15 minutes. The wounds were excised at various times after seeding and the tissue concentration of bacteria was determined.

The results showed that recovery of *S. aureus* per gram tissue declined with time after seeding. On the other hand recovery of *E. coli* did not decline during the first 165 min after seeding (Fig. 2).

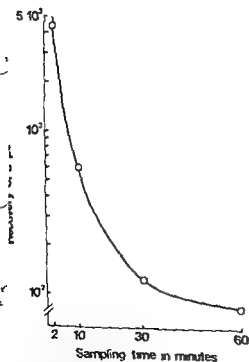


Fig. 1. Decline in recovery with time of *Staphylococcus aureus* from experimental wounds in pigs. Wounds were seeded with 1.2×10^5 *S. aureus* and sampling was performed with cotton swabs at 2, 10, 30 or 60 min after inoculation. Each entry is the mean of ten wounds.

Subsequent agar skin contact cultures were negative. A number of 4 cm incisions were made through skin and subcutaneous tissue. Bleeding was controlled by covering the wounds with sterile gauze for five minutes.

Staphylococcus aureus (Oxford strain 209, AB Bio disk) and four clinical isolates of *Escherichia coli* were used for the experiments. An overnight dextrose broth culture was diluted serially in saline and the number of colony forming units (CFU) was determined by viable count performed immediately before the experiments. Wounds were inoculated with 0.1 ml of bacterial suspension from one of the dilution tubes. The exact amount of bacterial suspension was administered to the wounds by micropipette and spread uniformly. Sampling was performed with a dry cotton swab (Torrent Corp.) which was moved backwards and forwards several times in the seeded wound and then streaked in a standard manner on a 9 cm blood agar culture plate. The number of CFU per plate was registered. When growth was heavy two representative areas on the plate were counted and the total growth estimated as described previously (Nyström 1978).

In one part of the study wound tissue concentration of bacteria was determined. The entire wound was excised and the skin was separated from the subcutane-

ous tissue and discarded. The remaining soft tissue weighed and diluted 1:10 (weight/volume) in phosphate broth, then homogenized with rotating knives. Homogenate was diluted serially and 0.1 ml of dilution was spread on blood agar culture plates. Colony forming units per gram tissue were calculated taking into account dilution, cultured volume and sample.

EXPERIMENTS AND RESULTS

Effect of Numbers of Seeded Bacteria on Recovery

This series of experiments was intended to determine the number of bacteria recovered as a function of the number of bacteria inoculated to define the lower limit for detection of infection.

In nine experiments with *Staphylococcus aureus* and four experiments with *Escherichia coli* 1.2×10^2 to 1.8×10^5 CFU in 0.1 ml were introduced into the wounds. Sampling was made after 10 min and the swab was streaked immediately onto culture plate.

TABLE 1. Quantitative Recovery by Swab Sampling 10 min after Inoculation of Experimental Wounds in Pigs with *Staphylococcus aureus*

Expt No	Inoculum per wound	No of wounds	Recovery per wound Mean \pm SEM CFU	Recovery as percentage of inoculum mean and range
1	1.2×10^2	10	0.8 ± 0.2	0.7 (0-1.7)
2	1.2×10^3	10	18.2 ± 1.5	1.5 (0.9-2.3)
3	1.8×10^3	10	13.7 ± 3.6	0.8 (0.06-1.9)
4	1.0×10^4	9	172.0 ± 33.3	1.7 (0.4-3.5)
5	1.2×10^4	10	82.4 ± 15.7	0.7 (0.09-1.3)
6	1.8×10^4	10	27.9 ± 5.1	0.2 (0.07-0.3)
7	1.2×10^5	10	423.4 ± 116.1	0.4 (0.05-1.0)
8	1.2×10^5	10	587.2 ± 91.7	0.5 (0.1-1.0)
9	1.8×10^5	9	248.9 ± 73.3	0.1 (0.06-0.5)

TABLE 2. Quantitative Recovery by Swab Sampling 10 or 30 min after Inoculation of Experimental Wounds in Pigs with *Escherichia coli**

Expt No	Time of sampling (min)	Inoculum per wound	No of wounds	Recovery per wound Mean \pm SEM CFU	Recovery as percentage of inoculum mean and range
1	10	1.0×10^4	9	638.4 ± 95.5	6.4 (1.7-12.1)
2	10	1.6×10^4	9	533.2 ± 59.8	3.3 (1.8-5.4)
3	10	1.8×10^4	10	533.6 ± 80.1	3.0 (1.4-5.2)
4	10	5.0×10^4	10	1836.2 ± 284.3	3.7 (1.6-6.8)
5	30	8.7×10^3	10	142.5 ± 20.0	1.6 (1.1-2.9)
6	30	1.1×10^5	8	2022.0 ± 361.2	1.8 (0.6-3.2)
7	30	1.1×10^5	10	2338.6 ± 240.8	2.1 (1.1-3.5)

* Four clinical isolates of *E. coli* were used in the experiments.

3BLF 4 Relative Efficacy of Three Different Swab Handling Procedures for Quantitative Recovery of *Staphylococcus aureus* from Experimental Wounds in Pigs

Expt No	Inoculum per wound	No of wounds	Swab procedures		
			Stuart ^a	Immediate ^b	Rinse ^c
1	1.8×10^3	10	1	1.5	7.0
2	1.8×10^4	10	1	1.5	2.0
3	1.2×10^5	10	1	3.6	4.1
4	1.8×10^5	9	1	2.8	3.5
		Mean	1	2.9	4.2

Swab stored for 18-24 hours in Stuart transport medium before streaking swab on culture medium

^a Streaking of swab on culture medium immediately after sampling

^c Rinsing of swab in 10 ml saline followed by plating of rinsing fluid immediately after sampling

for quantitative assessment of wound contamination in elective surgery

very few bacteria were recovered from the wounds there being a lower rate for *S. aureus* as compared to *E. coli*. The time lapse between contamination and sampling influenced the recovery rate whereas the size of the wound did not in the manner in which swabs were treated to release trapped bacteria onto the culture medium was important.

The findings will be discussed after a brief comment on the experimental model.

The sides of a wound had a surface area of

experiments. Wounds were inoculated with a small volume (0.1 ml) of bacterial suspension. A larger

volume rather than the wound tissue surface would have been sampled.

Although great care was taken to standardize all steps considerable variations in the results were encountered. Even within experiments coefficient of variation was high and occasionally reached 100%. In two experiments (not included) aimed at estimating the variation ten swabs were used simultaneously to sample one wound. Variation between swabs was 42% and 65% for the two experiments. A further analysis showed that 95% confidence limits of one and a half logarithms in either direction might be involved in predicting wound bacterial population from wound culture count. Variations in the same extent were apparently experienced by Scheibel *et al* (1978) in a similar experimental setting.

The low recovery of bacteria from the wound is

probably dependent on several factors and should be viewed as secondary in dynamic processes taking place in the wound the net result of which is exemplified in Fig 1.

A swab takes up 70% of a bacterial inoculum from an agar surface (Nyström 1978) but uptake is probably considerably lower from a wound due to bacteria being trapped in the rough wound surface.

Volume distribution i.e. dilution of bacteria in the wound fluid and passive or active penetration of the wound surface followed by peripheral dissemination of bacteria in the tissues begins within the first hour (Dhingra *et al* 1976).

The biological mechanisms of the interaction between bacteria and live tissue are extremely complex both due to the bacteria and to the host and these vary for different bacteria and different strains of the same bacterial species (Smith 1976, 1977). This may be exemplified by the differences in recovery of *S. aureus* and *E. coli* found in this study and by Scheibel *et al* (1978) with other micro-organisms.

Adhesion of bacteria to animal cells is considered to be a rapid process (Mardh & Nyström 1976, Bartels & Duncan 1978) and appears to be a characteristic of the bacteria in colonizing its specific biotope as well as a prerequisite for successful invasion of host by many bacterial pathogens (Sanage 1972, Gibbons & van Haute 1975, Eden *et al* 1976). Adhesion probably takes place in a wound as can be inferred from studies on wound cleansing by high pressure irrigation (Rodeheaver *et al* 1975, Hamer *et al* 1975, Brown *et al* 1978). In addition to adhesion there is also the possibility of aggregation or agglutination of bacteria in the wound thus producing microcolonies giving fewer colony forming units.

Specific immunological and non specific humoral

TABLE 3 *Influence of Wound Size on Quantitative Recovery of Staphylococcus aureus from Experimental Wounds by Swab Sampling*

Expt No	Inoculum per wound	No of wounds	Length of wound mean (mm)	Recovery per wound Mean \pm SEM CFU	Time of sampling (min)
1	1.0×10^4	9	34	172.0 ± 33.3	10
	1.0×10^4	10	85	144.2 ± 17.5	10
2	4.9×10^4	7	42	683.9 ± 183.6	10
	4.9×10^4	10	83	718.5 ± 111.8	10
3	5.1×10^4	10	41	463.3 ± 107.6	30
	5.1×10^4	10	95	339.1 ± 52.9	30

NS = not significant

Effect of Wound Size on Recovery

Using parallel experiments the influence of wound size was tested by comparing the standard wound of about 40 mm with a wound twice that length. Wounds in sets of ten for each size were seeded with *S. aureus* and sampled at 10 or 30 min.

Recovery from the bigger wound did not differ significantly from that of the smaller wound (Table 3).

Effect of Consecutive Sampling

To determine the significance of the first culture compared with a second or third culture from the

same wound, the following experiment was performed: one wound was seeded with 7.5×10^4 CFU and 10 min later the wound was sampled consecutive times.

A linear decline in recovery was observed in a semilogarithmic system (Fig. 3). Recovery was 0.4% for the first culture and cumulative recovery after ten cultures was 0.7%.

Effect of Different Swab Handling

This part of the study was aimed at determining the release of sampled bacteria from the swab by three different swab handling procedures.

Four sets of ten seeded wounds were sampled with three simultaneous swabs. One swab was immersed in modified Stuart's transport medium (Gasrini *et al.* 1968) kept at $+4^\circ\text{C}$ and streaked onto the culture plate the following day in order to mimic the procedure used in clinical practice for bacteriological specimens. The second swab was streaked onto the culture plate immediately after sampling. The third swab was placed in a tube with 10 ml saline. The tube was shaken on a vortex mixer for one min after which the swab was squeezed against the tube wall to remove as much fluid as possible and then discarded. The contents were emptied onto the culture plate which was rocked until the fluid covered the agar surface.

Immediate streaking gave a three times higher yield and the rinsing procedure a four times higher yield as compared to storage of the swab in Stuart transport medium (Table 4).

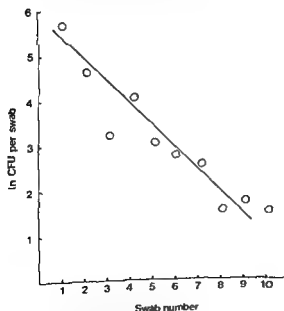


Fig. 3. Recovery using ten consecutive swabs from one wound seeded with 5×10^4 *Staphylococcus aureus* and sampled after 10 min. Recovery was 0.4% after the first swab and cumulative recovery after ten swabs was 0.7%.

DISCUSSION

The investigation was undertaken to determine the efficacy of sampling

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defence mechanisms as well as phagocytosis and intracellular killing of bacteria are probably operative within the first hour (Hohn *et al* 1977^b, Hohn *et al* 1977^a)

The low recovery of bacteria from the wound and the decline in recovery with time may then be viewed primarily as due to physical factors inherent in the sampling procedure as such and secondly due to adhesion of bacteria to the tissues, volume distribution and humoral and cellular inactivation and destruction of bacteria

Experimental investigations in animals (Edlich *et al* 1968, Kell 1978) and in man (Elek & Conen 1957) have demonstrated the high level of bacteria necessary to produce suppurative infection in undamaged tissue. Bacterial concentrations below 10^5 – 10^6 CFU seem to be eliminated without clinical infection. On the other hand quantification of the microflora in the wound in clean surgery (Burke 1963, Lilly *et al* 1970, Sanderson & Bentley 1976) and in potentially contaminated surgery (Taylor 1961, Raahave 1974, 1976, Nystrom in preparation) has demonstrated bacterial populations at the hundred level or lower and sometimes cultures are negative while the wound is subsequently found to be infected

Though potentiating factors such as sutures or ischemia which are always present to some extent in operation wounds reduce the number of bacteria necessary to produce suppuration (Elek & Conen 1957, Edlich *et al* 1968) there is still a discrepancy between experimental and clinical experience. Taking into account that the detection level in this study and in the work of Scheibel *et al* (1978) was in the range of 10^2 bacteria and higher as time elapsed after contamination this would appear to bring clinical results in accordance with experimental data

There is experimental and clinical evidence that post operative wound infection may occur subsequent to bacteraemia due to remote infection (Robson *et al* 1973). This might explain some of the infections in patients with negative incisional wound cultures

The cotton swab is commonly used for sampling microbiological specimens in clinical practice. Wound swabbing together with velvet pad imprint (Raahave 1974) and wound irrigation (Taylor 1961) are surface sampling methods as opposed to tissue biopsy homogenization (Arizek & Robson 1975) which is a volume sampling method. Surface sampling would appear to be the method of choice for per operative use in elective surgery (Raahave 1974)

Present evidence suggests that the recovery rate of bacteria does not differ much by the various

surface sampling methods but modifications aim at increasing the uptake of bacteria from wound or the collection of bacteria from sampler are important in increasing the sees of the methods (Raahave 1974, 1975a, b, Scheibel *et al* 1978, Nystrom 1978)

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STUDIES ON FUNGAL FLORA IN HAIR FROM DOMESTIC AND LABORATORY ANIMALS SUSPECTED OF DERMATOPHYTOSIS

1 Dermatophytes

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Aho R. Studies on fungal flora in hair from domestic and laboratory animals suspected of dermatophytosis 1 Dermatophytes Acta path microbiol scand Sect B 88 79-83 1980

Hairsamples of domestic and laboratory animals suspected of dermatophytosis were examined for the presence of dermatophytes. A nutritionally poor base medium developed by the author was successfully used in the isolation and identification of dermatophytes. Casein medium supplemented with vitamins and Sabouraud liquid medium were used in special cases. Dermatophytes were isolated in 36 of 331 samples (10.9%). The dermatophytes recovered were *Microsporum canis* 13 isolates from cat 4 from dog 1 from horse *Trichophyton mentagrophytes* var *granulare* 3 isolates from dog 3 from horse 2 from guinea pig and 1 from rabbit *Trichophyton terrestre* 1 isolate from dog. Eleven of the 13 feline isolates originated from house cats and the relative frequency was higher among the purebred cats. Two of the cat isolates were connected with human dermatophytosis.

Key words: Dermatophytes, fungal diagnostic medium, fungal zoonosis.

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Dermatophytosis, a fungal infection of the stratified layer of the skin, hair and nails is caused by dermatophytes of the *Microsporum*, *Trichophyton* and *Epidermophyton* families. According to Aulic (2) 21 *Trichophyton* and 15 *Microsporum* species are known of which at least 1 are pathogenic to man or animals or both. *Epidermophyton floccosum* is pathogenic to humans, is seldom reported to be pathogenic to animals (1).

A few reports have been published on animal dermatophytosis in Finland during the last three decades (16, 22, 23, 24).

The present investigation was undertaken to elucidate the occurrence and importance of dermatophytosis in domestic and laboratory animals in Finland and forms part of a more comprehensive study on fungal flora of skin in suspected cases of dermatophytosis.

MATERIAL AND METHODS

An appeal to veterinarians to send diagnostic material was published in the Journal of the Finnish Veterinary Association No 4 1977. The instructions were those suggested by Muller & Kirk (19). Veterinarians were

asked to send material to the Department of Microbiology and Epizootology at the College of Veterinary Medicine Helsinki. Of the 331 samples submitted (83%) were sent by 27 veterinarians from greater Helsinki (249 from small animals, 3 from laboratory animals, 23 from large animals). The 56 samples (17%) from other parts of Finland included 50 from southern Finland other than Helsinki, 3 from south-eastern Finland and 3 from western Finland (28 from small and 28 from large animals).

The source of samples according to animals species is given in Table 1. Nail as well as hair samples were

STUDIES ON FUNGAL FLORA IN HAIR FROM DOMESTIC AND LABORATORY ANIMALS SUSPECTED OF DERMATOPHYTOSIS

1. Dermatophytes

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Aho R. Studies on fungal flora in hair from domestic and laboratory animals suspected of dermatophytosis. 1. Dermatophytes. Acta path. microb. scand. Sect. B 88 79 83 1980

114 samples of domestic and laboratory animals suspected of dermatophytosis were examined for the presence of dermatophytes. A nutritionally poor base medium developed by the author was successfully used in the isolation and identification of dermatophytes. Casein medium supplemented with vitamins and Sabouraud liquid medium were used in special cases. Dermatophytes were isolated in 36 of 331 samples (10.9%). The dermatophytes recovered were *M. crosporum* ca. 13 isolates from cat, 4 from dog, 1 from horse, *T. choph. on menagroph. es. ar. granulare* 3 isolates from dog, 3 from horse, 2 from guinea pig and 1 from rabbit. *Tr. hoph. on. erc. re. 1* isolate from dog. Eleven of the 13 feline isolates originated from house cats and the relative frequency was higher among the purebred cats. Two of the cat isolates were connected with human dermatophytosis.

Key words: Dermatophytes, fungal diagnosis, medium, fungal zoonosis

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Dermatophytosis, a fungal infection of the keratinized layer of the skin, hair and nails, is caused by dermatophytes of the *M. crosporum* family and *Trichophyton* families. According to Aho (1) 21 *Trichophyton* and 15 *M. crosporum* species are known, of which at least 1 are pathogenic to man or animals or both. *Trichophyton mentagrophytes* is pathogenic to humans and is reported to be pathogenic to animals (1). A few reports have been published on animal dermatophytosis in Finland during the last three decades (16, 22, 23, 24).

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MATERIAL AND METHODS

An appeal to veterinarians to send diagnostic material was published in the Journal of the Finnish Veterinary Association No. 4 1977. The instructions were those suggested by Muller & Ark (19). Veterinarians were asked to wipe the coat with water or 70% ethanol, store the samples in a refrigerator and send them once a week to the laboratory. Samples were collected by veterinarians between April 1977 and May 1979 and sent to the Department of Microbiology and Epizootology at the College of Veterinary Medicine, Helsinki. Of the 331 samples submitted (83%) were sent by 27 veterinarians from greater Helsinki (249 from small animals, 3 from laboratory animals, 3 from large animals). The 56 samples (17%) from other parts of Finland included 50 from southern Finland other than Helsinki, 3 from south-eastern Finland and 3 from western Finland (28 from small and 28 from large animals).

The source of samples according to animal species is given in Table 1. Nails as well as hair samples were

TABLE 2 Age Specific Distribution of Dermatophytes in Dog and Cat

Dermatophytes isolated by age group																		
Animal	<1 year			1-2 years			2-4 years			>4 years			age unknown		total			
	n	+	%	n	+	%	n	+	%	n	+	%	n	+	%			
Dogs	17	2	11.8	42	3	7.1	45	1	2.2	98	2	2.0	4	0	0	206	8	3.9
Cats	13	7	53.8	18	2	11.1	8	2	25	20	2	10	2	0	0	61	13	21.3

TABLE 3 Sex Specific Distribution of Dermatophytes in Dog and Cat

Animal	Female			Male			Total			Sex unknown		
	n	+	%	n	+	%	n	+	%	n	+	%
Dogs	111	3	3.6	83	4	4.8	194	7	3.6	12	1	8.3
Cats	2	0	0	3	0	0	5	0	0			
Unsexed/sexed	25	4	16.0	32	8	25.0	57	12	21.1	4	1	25.0
Unsexed/sexed	5	0	0	9	0	0	14	0	0			

† Subgroup included in the group above

TABLE 4 Breed Specific Distribution of Dermatophytes in Cat

Cat	Occurrence of dermatophytes					
	Distribution by breed			In summary		
	n	+	%	n	+	%
Mixed short hair cats	39	5	12.8	39	5	12.8
Rebred cats	22	8	36.4			
Persian	15	5	33.3			
Colorpoint persian	1	0	0			
Siamese	2	1	50			
Burmese	2	1	50			
Rex	1	1	100			
Abernethian	1	0	0			
Total				61	13	21.3

rew also on the base medium but quickly growing contaminants soon interfered with the growth.

Microscopic examination of the native prepreparates did not prove worthwhile because of the rarity of spores in specimens from cases of dermatophytosis.

The positive cases originated in towns and villages of southern Finland except for one *M. canis* isolate from horse and three *T. verrucosum* isolates from cow which were sent from western Finland.

Of 32 animals treated with antifungal drugs before the examination two cats and one dog tested positive for dermatophytes.

In ten cases pets were examined mainly because of the pet owners' dermatophytosis. The skin infections of two pet owners (diagnosed by physicians of the Helsinki University Hospital) and their cats were culturally proved to be caused by *M. canis*. Six of the owners were demonstrated to suffer from

received from two dogs. No differences in quality of samples of small animals could be seen with the naked eye but approximately one third of the samples of large animals were distinctly dusty. This was verified by microscopic examination of native prepares.

Of the 206 dogs examined 188 were purebreds, 12 were mixed breed and 6 were of undesignated breed. The most common breeds were German shepherd (13 dogs), Westhighland white terrier (13 dogs), cocker spaniel (12 dogs) and dachshund (9 dogs). The breed distribution of cats can be seen in Table 4. Age and sex distribution of cats and dogs examined is displayed Tables 2 and 3.

According to information supplied with the specimens 12 cats, 18 dogs and 2 guinea pigs had been treated with local or systemic antifungal drugs in the two months before the fungal examination. In ten cases the pet owner was suspected of having dermatophytosis and the pet was examined mainly for this reason. All these animals were reported to show at least slight symptoms. Some of the animals examined apparently had other diseases: seven dogs and three cats had symptoms of hormonal imbalance, one dog diabetes mellitus, one dog acanthosis nigricans and four dogs demodicosis.

Native preparations of each sample were mixed with potassium (5 g), glycerine (25 ml), distilled water (20 ml) solution and applied to the object glass and examined by a phase contrast microscope. About twenty hairs of each sample were cultivated on a base medium developed by the author (5 g glucose, 5 g peptone, 15 g agar, 1000 ml distilled water, 20 iu/ml penicillin, 40 iu/ml streptomycin, pH = 6.6). Nail samples were kept 10 minutes in 70% ethanol then cut into small pieces and mounted on the base medium. Besides the base medium hair samples of cow were also mounted on Casein thiamine inositol agar (8) and Sabouraud liquid medium (25) and hair samples of horse on Casein niacin acid agar (8). The incubation temperature was +22 °C except for the cow

hair cultures which were incubated at both +2 +35 °C. The laboratory was equipped with 15 Plates and liquid medium cultures were incubated 6-8 weeks if necessary and examined every first

RESULTS

The distribution of the material by animals and the dermatophyte isolates by species is listed in Table 1. Dermatophytes were found in 10.9% of the hair samples (10.9%). The two nail samples examined were negative. The age and sex frequencies of feline and canine dermatophytes are given in Table 2 and 3. The breed distribution of dermatophytosis in cats can be seen in Table 4. Dermatophytes of dogs were isolated from seven purebred dogs (twice in dachshund, once in Keeshond, German shepherd, for doberman and boxer) in one positive case it was not known. Fifty nine of the 61 cats examined were house pets. The two strays examined were positive.

The nutritionally poor base medium was fully used in isolation and identification of *M. T. mentagrophytes* and *T. terrestre*. The growth of these species was detected within 4-7 days. Typical spores and pigments were produced in 5-14 days. Contaminant fungi e.g. *Aspergillus*, *Penicillium* species grew slower than in the naturally rich Casein agar. *T. verrucosum* (8%) was most successfully isolated on Sabouraud medium and in one case only on Casein thiamine inositol medium. *T. verrucosum* as a slow grower

TABLE 1 Occurrence of Dermatophytes in Hair Samples by Species and Host

	Number of animals examined	Microsporium canis	Trichophyton mentagrophytes	T. verrucosum	T. terrestre	Percent of pos.
Dog	206	4	3	-	1	3.9
Cat	61	13	-	-	-	21.3
Horse	27	1	3	-	-	14.8
Cow	19	-	-	8	-	42.1
Guinea pig	10	-	2	-	-	20.0
Rabbit	1	-	1	-	-	100.0
Cage bird	2	-	-	-	-	-
Goat	1	-	-	-	-	-
Chinchilla	1	-	-	-	-	-
Rat	1	-	-	-	-	-
Mink	1	-	-	-	-	-
Lesser panda (<i>Ailuropus fulgens</i>)	1	-	-	-	-	-
	331	18	9	8	1	10.9

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T. mentagrophytes infection, but the same agent could not be isolated in the hair samples of their pets. In two suspected cases, fungal examination proved negative both in humans and animals.

In four cases of *T. verrucosum* isolation in cattle, an outbreak of dermatophytosis on the farm was clinically reported. A *M. canis* infection in horse was connected with a stable epidemic of dermatophytosis, apparently introduced by a five-year-old stallion and spreading to two foals.

DISCUSSION

The nutritionally poor base-medium was developed by the author with the idea that such a medium would favour the development of the fruit bodies which are valuable to the identification of the genera (7). From this point of view the medium proved to be successful (unpublished preliminary results). To what extent the lack of nutrients in the medium influence the spectrum and proportion of dermatophytes and contaminants cannot be more exactly estimated before comparative studies have been carried out. However, dermatophytes which need vitamin addition for quick growth, e.g. *T. verrucosum* presumably are unable to compete successfully with quickly growing fungi. The addition of cycloheximide to the base-medium might also be of value in preventing the overgrowth of contaminants. This will be studied in the future.

Whether and to what extent the method applied, especially the collection and handling of samples led to false negative findings is one of the most important considerations as regards the reliability of the results. The great amount of contaminant fungi in some of the specimens, especially those from large animals, must have had a preventive influence upon the growth of dermatophytes. Fast growing fungi which additionally may possess antagonistic properties greatly inhibit the growth of slowly growing species. The time between obtaining the sample and cultivating it was less than two weeks which probably had no influence on the viability of dermatophytes (12-19). But many contaminants could have grown during the mailing period suppressing the growth of the dermatophytes.

The collection of samples from animals with only slight symptoms would have been better carried out by the Mackenzie hairbrush-technique (3).

Antifungal treatment of animals before examination naturally may have influenced the results in respective cases. However Wright (26) has presented evidence that even after 8 weeks treatment with griseofulvin, isolation of dermatophytes may still be possible.

According to Muller & Kirk (19) young most frequently suffer from dermatophytosis. Our material only 8.3% of dog hair samples, 21.3% of cat hair samples came from animals less than one-year old, but the highest incidence of dermatophytosis was nevertheless found in this age group. Sex disposition could be statistically proved in dermatophytosis of dog and cat, but dermatophytosis was found in 36.4% of purebred cats compared with 12.8% of domestic short-haired cats examined (χ^2 -test $p < 0.05$). This may be due to the contacts with other cats in cat shows, mating, etc. Kushida (14) has noticed a breed disposition. No significant breed disposition was found in relative frequency of positive dog isolates.

The results of this study are in the main in agreement with those published earlier (3, 4, 6, 10, 11, 15, 17, 18, 20, 22, 23, 24, 26). Sonck reported *M. canis*-epidemics in outdoor cat in Turku (south-western Finland). In the present study based on a different approach, 11 of the 13 feline dermatophytosis were found in home-bred cats. All the cases of canine dermatophytosis were found in family-dogs. Indoor pets especially purebred cats and dogs, have elsewhere been reported to be infected by *M. canis* (4, 10, 11, 14) and has been considered the most common and important zoonosis dermatophyte in cats (1). The zoonosis character of *M. canis* infection needs additional support from the present results. Failure to demonstrate *T. mentagrophytes* in pets of 6 verified human cases even with the least slight symptoms of skin disorder reported may reflect either negativity of the source of infection or nonsensitivity of the isolation procedure.

The importance of *T. terrestris* in dermatophytosis has been discussed by several authors. Although this species has generally been considered an apathogenic, geophilic dermatophyte, Gip (9) and Connole (5) have reported its role to cause ringworm-like lesions. Salonen reported it in Finnish soil. In this study on *terrestris*-isolation was made and its pathogenic significance is uncertain.

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T. mentagrophytes infection but the same agent could not be isolated in the hair samples of their pets. In two suspected cases fungal examination proved negative both in humans and animals.

In four cases of *T. verrucosum* isolation in cattle an outbreak of dermatophytosis on the farm was clinically reported. A *M. canis* infection in horse was connected with a stable epidemic of dermatophytosis apparently introduced by a five-year-old stallion and spreading to two foals.

DISCUSSION

The nutritionally poor base medium was developed by the author with the idea that such a medium would favour the development of the fruit bodies which are valuable to the identification of the genera (7). From this point of view the medium proved to be successful (unpublished preliminary results). To what extent the lack of nutrients in the medium influence the spectrum and proportion of dermatophytes and contaminants cannot be more exactly estimated before comparative studies have been carried out. However dermatophytes which need vitamin addition for quick growth e.g. *T. verrucosum* presumably are unable to compete successfully with quickly growing fungi. The addition of cycloheximide to the base medium might also be of value in preventing the overgrowth of contaminants. This will be studied in the future.

Whether and to what extent the method applied especially the collection and handling of samples led to false negative findings is one of the most important considerations as regards the reliability of the results. The great amount of contaminant fungi in some of the specimens especially those from large animals must have had a preventive influence upon the growth of dermatophytes. Fast growing fungi which additionally may possess antagonistic properties greatly inhibit the growth of slowly growing species. The time between obtaining the sample and cultivating it was less than two weeks which probably had no influence on the viability of dermatophytes (12-19). But many contaminants could have grown during the mailing period suppressing the growth of the dermatophytes.

The collection of samples from animals with only slight symptoms would have been better carried out by the Mackenzie hairbrush technique (3).

Antifungal treatment of animals before examination naturally may have influenced the results in respective cases. However Wright (26) has presented evidence that even after 8 weeks treatment with griseofulvin isolation of dermatophytes may still be possible.

According to Muller & Kirk (19) young animals most frequently suffer from dermatophytosis. In our material only 8.3% of dog hair samples and 21.3% of cat hair samples came from animals less than one year old but the highest incidence of dermatophytosis was nevertheless found in the sex disposition could be statistically proved. Dermatophytosis of dog and cat but dermatophytosis was found in 36.4% of purebred cats etc. compared with 12.8% of domestic shorthairs examined (χ^2 test $p < 0.05$). This may derive from the contacts with other cats in cat shows, grooming etc. Aushida (14) has noticed the influence of breed disposition. No significant breed difference in relative frequency of positive dog isolates was found.

The results of this study are in the main in accordance with those published earlier (3, 4, 6, 10, 11, 15, 17, 18, 20, 22, 23, 24, 26). Sorek (7) reported *M. canis*-epidemics in outdoor cats in Turku (south western Finland). In the present study based on a different approach 11 of the 13 canine feline dermatophytosis were found in house pets. All the cases of canine dermatophytosis were found in family-dogs. Indoor pets especially bred cats and dogs have elsewhere been reported to be infected by *M. canis* (4, 10, 11, 14) and *M. canis* has been considered the most common and important zoonosis dermatophyte in cats and dogs. The zoonosis character of *M. canis* infection is supported by additional support from the present results. Failure to demonstrate *T. mentagrophytes* in pets of 6 verified human cases even the least slight symptoms of skin disorders reported may reflect either negativity of the source of infection or nonsensitivity of the isolation procedure.

The importance of *T. terrestre* in canine dermatophytosis has been discussed by several authors. Although this species has generally been considered an apathogenic geophilic dermatophyte, Gip (9) and Connole (5) have reported its capacity to cause ringworm like lesions. Salonen (2) reported it in Finnish soil. In this study only *terrestre* isolation was made and its pathogenic significance is uncertain.

The Finnish Foundation for Veterinary Medicine supported the work with a grant.

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EXPERIENCE WITH AN INDIRECT (PASSIVE) HAEMAGGLUTINATION TEST FOR THE DEMONSTRATION OF RUBELLA VIRUS ANTIBODY

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J. Haukenes Experience with an indirect (passive) haemagglutination test for the demonstration of rubella virus antibody Acta path microbiol scand Sect B 88 85-87 1980

A new indirect (passive) haemagglutination technique (Rubacell Abbott) has been evaluated on the basis of a material of 1 792 sera sent for serological diagnosis of a rubella infection or determination of immunity status. The test procedure is very simple. IHA antibodies appeared about two weeks later than HI antibodies and seemed to persist for life. Two fields of application for the IHA technique are discussed: 1) As a supplement to other tests because of the late appearance of the antibodies. 2) In screening for immunity status. The IHA combines ease of performance with high sensitivity.

Key words: Rubella, indirect haemagglutination, passive haemagglutination.

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Rubella infection is usually diagnosed serologically by demonstration of seroconversion or a rise in titre by the haemagglutination-inhibition (HI) test, the haemolysis in gel (HIG) test or one of the tests using labelled anti-immunoglobulin. The reactive antibodies can be detected a few days after appearance of the rash. In most cases the titre reaches a maximum level within one week, somewhat with the complement fixation test. If the first serum specimen is taken in the second week of disease or later, a rise in titre may often not be demonstrable. In such cases the diagnosis can be made only by demonstrating specific IgM. An indirect (passive) haemagglutination (IHA) test has been developed by Abbott Laboratories. The antibodies detected by this IHA persist after a rubella infection (4). Meurman (3) collected several serum specimens from 31 patients with acute rubella and compared the IHA with other tests. He concluded that the IHA antibodies appeared two to three weeks later than HI antibodies, so that the finding of a positive HI and a negative IHA was diagnostic of an acute rubella infection and made demonstration of IgM unnecessary.

We have examined the IHA test on a material consisting of about 1 800 sera in order to evaluate its reliability and usefulness.

MATERIALS AND METHODS

Sera

The sera were sent to the laboratory for diagnosis of rubella infection or determination of immunity status. They were inactivated and examined either fresh on arrival or after storage at -20 °C.

Serological Tests

The standard HI test was performed after absorption of the serum with kaolin at pH 9 and using sheep erythrocytes. A newly developed HI procedure, not requiring removal of non-specific inhibitors (2), was run parallel with the standard test. In brief, sheep erythrocytes were sensitized by four haemagglutination (HA) units of the haemagglutinin. When the sensitized erythrocytes were mixed with an antiserum, antibody inhibited the HA without interference by non-specific inhibitors. The IHA (Rubacell Abbott Labs) was performed as described by the manufacturer. Twenty-five µl serum diluted 1:135 was mixed with 25 µl stabilized human erythrocytes sensitized by a soluble rubella virus antigen using microtitre V-plates. The plates were tapped, shaken

for a few minutes and left at room temperature for 2 h or more before reading. The IHA patterns were distinctly negative or positive unless the sera had been taken during the first weeks after an acute rubella infection at the time of seroconversion. Because of the small volume of the reactants (50 µl) only a limited number of sera should be set up before taping in order to avoid evaporation.

Haemolysis in Gel (HIG)

This was performed on commercial plates (Orvir Orion Diagn. Helsinki). A haemolysis zone of 5 mm or more was considered as a positive reaction (5).

Flotation Centrifugation

This was performed as described in (1) in cases where it was uncertain whether a very weak HI reaction was caused by antibody or inhibitor.

RESULTS

A total of 1792 sera were examined by three parallel methods: standard HI, new HI and IHA. The IHA was performed qualitatively at dilution 1:135 unless a rise in titre in connection with a recent rubella was suspected. The results obtained by the new HI procedure have been published elsewhere (2). They correlated almost completely with the results obtained by the standard HI test.

All readings were made blind i.e. without knowledge of the history or the results of other tests. When in doubt the IHA was repeated and often titrated. When the test was repeated because of any disagreement only the first reading was recorded in order to evaluate the test as a single screening method for immunity status.

When there was any disagreement between IHA and the HI tests an HIG test was performed and occasionally also floating centrifugation.

When acute rubella infection was suspected the serum specimens were taken at the intervals recommended for the HI test. If the second specimen was still IHA negative a third specimen was requested even when the diagnosis had been established. The results are shown in Table 1. Fourteen sera were HI negative and IHA positive. Six of these gave a false positive IHA reaction as defined by a negative HI and HIG test. The six sera had been examined during a period when we had considerable difficulty in interpreting the IHA pattern. They were re-examined with a new batch of sensitized cells and were all HI & negative. The other eight of the sera which showed positive IHA and negative HI showed a false negative HI reaction.

The group negative IHA and positive HI comprised 131 sera, 113 of which were from patients with an acute or recent rubella infection.

TABLE 1 Comparison of HI and IHA

Positive HI and IHA	1
Negative HI and IHA	
Negative HI and positive IHA	1
False positive IHA	6 ^a
False negative HI	8 ^b
Positive HI and negative IHA	1
Acute rubella	113
False negative IHA	6 ^b
False positive HI	4 ^c
Unidentified	8 ^d

Total number of sera examined

- a Negative HIG
- b Positive HIG
- c Seroconversion later
- d Suspected rubella. One serum specimen only

They all showed seroconversion later. Eight were listed as non-identified. They were from suspected rubella but the diagnosis was not verified since we received only one serum. Six sera gave a false negative IHA reaction. When re-examined they were found to be positive. Four sera gave a false positive HI reaction.

All sera were collected during a rubella epidemic and pregnant women had usually consulted a doctor immediately when they had been exposed to rubella or suspected a rubella infection. Only a few sera were taken too late to obtain a rise in titre by means of the HI test. In our routine we examine the first serum sample for IgM when it is taken from pregnant women with a history of rubella up to 6 months earlier. Only in two cases could the diagnosis be made by seroconversion in HI stationary HI titres.

We tried to calculate when the IHA was positive in relation to the first day of the rash. In 106 patients with acute rubella we obtained information about the date of the rash. The time from the first day of the rash to the first negative IHA test was 14 days (range 3 to 33). The average time for the first positive IHA was 45 days (range 5 to 68 days). The average negative period was somewhere between 14 and 24 days.

When performed as a qualitative test the procedure is very simple and a trained technician can perform about 50 tests per hour.

DISCUSSION

The new IHA method developed by the Laboratories represents a new and valuable addition to the serological diagnosis of rubella.

larly for the assessment of immunity status suits with a material of about 1 800 sera ■ the findings obtained by *Meurman* (3) from 1 material studying IHA in the diagnosis of rubella Laboratories which receive a great nr of specimens will possibly resort to one of thods which can be automatized such as RIA LISA both when examining for immunity ise or specific IgM Other laboratories will to choose between HIG and one of the H ques for the diagnosis of rubella infection

Logical Diagnosis of a Rubella Infection

e IHA should never be used as the only test for dy when a rubella infection is suspected As a ement to the other tests which become positive days after the appearance of the rash the IHA give valuable information even at the time of x of the first serum specimen A positive HI RIA or ELISA combined with negative IHA hly indicative of an acute rubella infection The osis must of course be confirmed by a rise in or by demonstration of rubella specific IgM a false negative IHA reaction may occur ever since acute rubella is suspected already n the first serum specimen is examined rmatory tests may be performed immediately unnecessary delay is avoided The advantage of ■ a supplementary test is thus obvious If a ratory does not have the capacity for examining antibody positive first serum specimens from nant women for IgM the IHA should be rmed in the absence of a rise in titre

rubella reinfection is suspected when a rise in is found without the presence of IgM and re there are no symptoms In two cases not uded in this material we suspected a reinfection these grounds The finding in both of them of a n and stable IHA titre at the time when the HI I HIG titre was still rising in the absence of IgM e further support to our suspicion Later gnosis of a reinfection was verified for one of m when we obtained a serum sample that had n taken half a year earlier If the titre obtained using the other tests has elled off the IHA may still show seroconversion a rise in titre Our findings agree with those uined by *Meurman* (3) as regards the average

of the

Screening for Immunity Status

The performance of the IHA test is very simple d the price is reasonable IHA antibodies seem to for life The number of false positive

reactions was low This could be related to a special batch of erythrocytes and such reactions have not been recorded since It has been argued that the late appearance of IHA antibodies is a drawback when screening for immunity status Obviously very few individuals can be proved to be in the two to three weeks seronegative period after an asymptomatic rubella infection and the vaccine will certainly do them no harm Another question ■ whether a quantitative method might pick up individuals with very high serum titres thus suggesting a recent infection and leading to examination for specific IgM This is of interest only when women are examined in the first trimester of their pregnancy It is difficult to predict the results of such a practice We have titrated rubella HI antibody in sera taken both early and late in pregnancy from about 2 000 women (unpublished) Three cases of seroconversion and one reinfection were found Nineteen sera with HI titres of 640 or higher were examined further by HIG and IHA and for specific IgM No signs of recent infection were detected It must be emphasized however that the sera were collected during an inter-epidemic period just before the start of an epidemic It remains to be seen whether the IHA may prove useful in this context The HA pattern may be very weak or doubtful for a considerable period ■ the time of seroconversion

In our opinion screening for rubella immunity by means of this IHA should be performed only in laboratories that carry out serological diagnosis of rubella infection where IHA is included Only in this way can the reagents for the IHA test be checked regularly for sensitivity and reliability If IHA is used to supplement another method of screening for immunity status recent infections may also be revealed Such screening will be carried out in our laboratory to evaluate further the place of the IHA in this context

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CELLULAR FATTY ACID COMPOSITION OF *HAEMOPHILUS* SPECIES, *PASTEURELLA MULTOCIDA*, *ACTINOBACILLUS ACTINOMYCETEMCOMITANS* AND *HAEMOPHILUS VAGINALIS* (*CORYNEBACTERIUM VAGINALE*)

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Jantzen E, Berdal B P & Omiland T. Cellular fatty acid composition of *Haemophilus* species *Pasteurella multocida*, *Actinobacillus actinomycetemcomitans* and *Haemophilus vaginalis* (*Corynebacterium vaginae*). Acta path microbiol scand Sect B 88 89-93 1980

The fatty acid composition of 35 *Haemophilus influenzae* strains was found to be grossly similar and characterized by relatively large amounts of 14:0 3-OH 14:0 16:1 and 16:0. The three C₁₈ fatty acids 18:2 18:1 and 18:0 were also present but in much lower concentrations. This general pattern was also found for most of the other species of *Haemophilus* examined (*H. aegyptius*, *H. aphrophilus*, *H. canis*, *H. gallinarum*, *H. haemolyticus* and *H. parainfluenzae*). Small but distinct quantitative discrepancies were detected for *H. ducreyi* and the haemin independent species *H. paraphaemolyticus*, *H. paraphrophilus* and *H. suis*. *Actinobacillus actinomycetemcomitans* was found to be indistinguishable from *H. influenzae*. *Pasteurella multocida* also exhibited a fatty acid pattern closely related to that of *Haemophilus* but could be distinguished by its higher concentration levels of the C₁₈ fatty acids. The fatty acid pattern of *H. vaginalis* was considerably different from those of the other species examined. This species lacked 3-OH 14:0 and 18:2 and contained small amounts of 14:0 and 16:0 whereas 18:1 and 18:0 were the major constituents.

Key words: Bacteria, fatty acid composition, gas chromatography, taxonomy.

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Numerous taxonomic studies based on bacterial fatty acid composition have revealed clearly the usefulness of quantitative data on lipid constituents in taxonomic evaluations (1-13, 14-17). Fatty acid profiles provide simultaneously several taxonomic characters and it has been shown that grouping based on fatty acid composition matches reasonably well the classification achieved by genetic parameters (5-7). However, before this chemical technique can be fully utilized in taxonomy, many groups of well characterized bacteria and if possible several strains of each species must be analysed preferably by a standardized procedure.

We are considering in particular the use of fatty acid profiles in the identification of unusual clinical

isolates which are difficult to assign to genus or species and have atypical fatty acid patterns.

This paper deals with the fatty acid composition of several *Haemophilus* species and of *Pasteurella multocida*. Two strains of *Actinobacillus actinomycetemcomitans* and one strain of *H. vaginalis* (*Corynebacterium vaginae*) were also included in the study since their relatedness to *Haemophilus* appears to be unclear at present. The fatty acid composition of *Francisella tularensis* has been published elsewhere (8) and studies of *Bordetella* and *Brucella* are in progress.

MATERIALS AND METHODS

Bacterial Strains, Growth and Harvesting

Thirty-five *Haemophilus influenzae* strains were analysed. Fourteen were obtained from the National Collection of Type Cultures, London, designated by the following NCTC numbers 4842, 7279, 7918, 8143, 8455, 8465, 8466, 8467, 8468, 8469, 8470, 8472, 8473, 10479. Six serological reference strains (Omland), A51, B51, C51, D51, E51 and F51 were received originally (1957) from H. C. Engbak, Statens Serum Institut, Copenhagen, and two strains (E-Montenegro and F-Dingles) from G. Leidy, Columbia University, NY. The remaining thirteen strains were own isolates.

In addition, the following *Haemophilus* species were examined: *H. aegyptius* NCTC 8134, NCTC 8502, *H. aphrophilus* NCTC 5886, NCTC 5906, *H. canis* NCTC 1659, NCTC 10619, *H. gallinarum* NCTC 3438, *H. haemolyticus* NCTC 8479, NCTC 10659, *H. parainfluenzae*, NCTC 7857, NCTC 7857, NCTC 10665, *H. paraphrohaemolyticus* NCTC 10670, NCTC 10671, *H. paraphrophilus* NCTC 10556, NCTC 10557, *H. suis*, NCTC 4557, NCTC 6359, *H. ducreyi* ATCC 27722, and one strain received from K. Odegaard, National Institute of Public Health, Oslo.

The two *Actinobacillus actinomycetemcomitans* strains NCTC 9709, NCTC 9710 and the single representative of *H. vaginalis* NCTC 10287 were obtained from the National Collection of Type Cultures.

Of the sixteen *Pasteurella multocida* strains examined, fifteen were received from S. D. Henriksen, University of Oslo. These were serological reference strains received originally from Japan: Kobe 5 (1 A), M4 (1 A), Kobe 6 (2 O), P 27 (2 O), P 8 (3 A), M17 (4 O), TS 8 (5 O), VA 3 (5 A), R 4 73 (6 B), Buma (6 E), PM (7 A), 147 (8 ?), Liver (9 A) and 989 (11 B). One strain, NCTC 3195, was obtained from the National Collection of Type Cultures.

The strains were maintained and cultivated on chocolate agar (10% heated defibrinated horse blood in blood agar base (Difco)). One single batch of medium was used for cultivation. Incubation was performed for 24 hours at 37 °C in the presence of 5% CO₂. The bacteria were harvested in phosphate buffer (0.5 M, pH 7.2), centrifuged and washed twice by distilled water before freeze drying.

Chemicals and Chemical Analyses

Solvents of pro analysis grade were distilled before use. Standards of fatty acid methyl esters were obtained from Applied Science Laboratories Inc., State College, Penn., USA.

Dried bacterial cells (1–10 mg) were methanolized by 2 N HCl in dry methanol as described previously (6, 7). Fatty acid methyl esters were analysed on a Hewlett Packard 5710 gas chromatograph equipped with a flame ionization detector. Two standard 2 m glass columns packed with either 10% SE-30 or CP-10 on Gas Chrom Q 100–120 mesh (Applied Science Laboratories Inc.) were used routinely. Additional analyses were performed

on a 25 m SCOT SE-30 Column (F. Middelburg, The Netherlands).

Peak identifications were obtained by retention data with those of authentic standards. Columns: Hydroxy fatty acids were trifluoroacetylation and rechromatography (7). Natural elucidations were verified by gas chromatography mass spectrometry on a Hewlett Packard 1 chromatograph mass spectrometer instrument with a 2 m long 1% OV-1 glass column.

RESULTS

All thirty-five *H. influenzae* strains grossly similar fatty acid patterns with relatively large amounts of 14:0 (see Table 1 nomenclature), 3-OH-14:0, 16:1 and 18:1 and three C₁₈ fatty acids, 18:2, 18:1 and 18:0 generally present but in much lower (Table 1). All recognized serotypes (a to Pittman scheme) and also the biotypes Kilian (11) were represented. However, variation between fatty acid composition and/or biochemical characteristics could be

The fatty acid pattern found for *H. suis* was shared by the other haemophilus except for *H. ducreyi* which differed slightly higher concentration of 14:0 and a lower concentration of 16:0. Species of the haemophilus group constituted a more heterogeneous group. *H. aphrophilus*, *H. gallinarum* and *H. parainfluenzae* were almost indistinguishable from the requiring species, whereas *H. paraphrophilus*, *H. paraphrophilus* and *H. suis* show discrepancies in the 14:0 and 16:0 concentration comparison to the *H. influenzae* pattern.

A. actinomycetemcomitans could not be distinguished from *H. influenzae* or *H. aphrophilus*. *P. multocida* exhibited a fatty acid pattern that of *Haemophilus* (Table 1). However, as indicated in Fig. 1, this species could be distinguished by a higher level of the C₁₈ fatty acids. On the other hand, as could be expected, *H. vaginalis* exhibited a very different pattern. It lacked 3-OH-14:0 and 18:2 and contained small amounts of 14:0 and 16:1, whereas 18:1 and 18:0 were the most abundant fatty acids.

DISCUSSION

In an extensive taxonomic study of genus *Haemophilus* (11), Kilian recently showed the G+C content of DNA to be in the range of 37 to 44 mole per cent. All haemophilus species exhibited a G+C content below 40 mole per cent, whereas the haemophilus-independent

1 Percentage Cellular Fatty Acid Composition of *Haemophilus* Species: *Actinobacillus actinomycetemcomitans*
Haemophilus vaginalis (*Corynebacterium vaginale*) and *Pasteurella multocida*¹

	%G + C of DNA ²	ALA ↑ porphyrin ³	V factor requirements ⁵	Fatty Acid						
				14:0 ⁶	3-OH 14:0	16:1	16:0	18:2	18:1	18:0
<i>aer</i> (ns)	38.7	-	+	12.75 8.6-16.9 ⁶ 2.30 ⁷	11.6 9.9-13.4 1.08	31.4 24.3-35.5 2.41	40.6 36.4-46.5 2.41	0.2 0.0-0.5 0.15	0.5 0.2-0.8 0.16	2.4 1.1-5.6 1.13
<i>us</i>	36.1	-	+	16.2 13.8	12.8 14.8	28.0 24.5	39.9 40.5	0.2 0.4	0.5 0.4	2.4 4.4
<i>h. lus</i>	42.0	+	-	15.8 23.0	14.7 13.8	26.3 22.7	38.7 38.0	0.4 0.3	0.4 0.4	2.7 1.3
	38.1	-	-	17.4 13.5	13.3 13.8	24.7 24.7	40.1 40.6	0.2 0.7	1.1 0.8	2.9 4.9
<i>rum</i>	42.2	+	+	10.7	12.4	29.1	41.3	0.5	0.7	5.6
<i>ip. cus</i>	38.5	-	+	23.2 12.4	17.7 23.8	36.5 33.4	28.4 40.3	0.4 1.0	0.9 2.1	1.4 9.3
<i>fluor. pe</i>	39.7	+	+	13.3 12.7	13.8 16.2	32.0 33.8	36.7 33.1	0.6 0.7	1.0 1.1	2.1 2.0
<i>hro</i> <i>cus</i>	40.6	+	+	34.3 43.0	17.6 17.3	21.9 19.6	16.8 18.2	0.2 0.4	0.5 0.5	0.5 0.6
<i>hroph. lus</i>	41.9	+	+	22.0 21.7	14.4 15.0	26.6 26.7	33.9 34.6	0.6 0.3	1.0 0.8	0.9 0.7
	41.5	+	+	8.6 6.7	12.8 11.1	33.0 42.7	35.4 26.1	1.0 0.6	1.2 6.5	6.7 5.7
<i>us</i>	37.8	-	-	24.8 25.4	10.2 10.8	35.3 34.1	26.3 24.7	0.3 0.4	1.0 0.9	0.8 0.9
<i>actinomycetem</i>	42.7	+		12.2 16.7	18.1 15.0	26.9 24.6	37.3 38.4	0.3 0.3	0.7 0.9	3.1 2.1
<i>tal. s</i>	42 ± 1 ⁸			4.4	-	2.0	54.8	-	27.3	10.4
<i>ac. da</i> (ns)	36.5-40.1 ⁸	+	-	10.75 8.0-12.8 ⁶ 1.31 ⁷	12.9 12.2-14.7 0.77	29.3 23.0-32.0 2.07	38.7 36.7-42.0 1.43	1.9 1.0-3.4 0.61	1.7 0.7-3.0 0.50	4.6 3.5-6.4 0.83

See text for experimental details

See text for further description

Data of Kihara (11)

Key to the fatty acid designation: the figure in the front of the colon indicates the number of carbon atoms in the chain; the figure after the colon, the number of double bonds; the symbol 3-OH indicates a hydroxy group and ns, position.

Mean value

Range among strains

Standard deviation

Data of Lapage (12)

Data of Beluversky & Spirin (2)

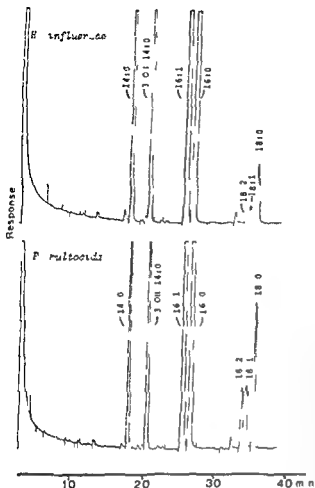


Fig. 1 Typical gas chromatographic fatty acid methyl ester profiles of a *Haemophilus influenzae* and a *Pasteurella multocida* strain.

The bacterial cells were digested by 2 N HCl in methanol, extracted and trifluoroacetylated before injection on the 25 m SCOT SE 30 column.

G + C values above that level. These two main groups of *Haemophilus* could not be revealed clearly by the fatty acid patterns. Thus all haemin requiring species except *H. ducreyi* and the two haemin independent species *H. aphrophilus* and *H. gallinarum* shared the *H. influenzae* pattern whereas the remaining four haemin independent species showed small but significant deviations both from each other and from *H. influenzae* (Table 1).

Previous results have shown that only species of a certain phylogenetic distance can be distinguished by their fatty acid composition. Within *Moraxella* most species could be differentiated (7). On the other hand within the *Enterobacteriaceae* where speciation is based mainly on serological characteristics only a few groups could be differentiated (9). The taxonomic distances between the *Haemophilus* species are apparently too small to be clearly reflected by their fatty acid compositions.

In the latest edition of Bergey's Manual (4) the

species *A. actinomycetemcomitans* is excluded from the genus *Actinobacillus* and listed as species *sedis* (16). Ailian (11) demonstrated a difference of this species to *H. aphrophilus* and discussed the possibility of its inclusion in genus *Haemophilus*. We were not able to distinguish *A. actinomycetemcomitans* from *Haemophilus* by our method. The fatty acid data support a taxonomic revision in this direction.

The taxonomic position of *H. vaginalis* is also questionable. As pointed out by Lapage in Bergey's Manual this species does not belong to the genus *Haemophilus*. Its association with the *Campylobacter* genus, as suggested by Zinner and Turner (19), is also unlikely since its cell wall contains dimethylsuccinic acid and contains 6-deoxyarabinose. Lapage accordingly recommends retaining the name *H. vaginalis* without changing its appropriate taxonomic position for this order. This was revealed. In terms of fatty acid composition, this species differed from most Gram-negative bacteria by the absence of hydroxy fatty acids (13). The fatty acid pattern of *H. vaginalis* was also different from that reported for *C. diphtheriae* and *C. acnes* as well as from a group of pleomorphic soil bacteria (*C. fascians*, *C. michiganense*, *C. sedgwickii*, *C. uniusdosum*) (15). These two groups were characterized by branched fatty acids as major constituents. Further comparative studies of fatty acid composition may assign *H. vaginalis* to a different genus.

Haemophilus, *Pasteurella*, and *Actinobacillus* share a number of phenotypic characters and their G + C contents are in the same range (10). In a numerical taxonomic study including representatives of these three genera, Sneath & Johnson (1) obtained four clusters of relatively high correlation coefficient. On this basis the authors suggested that these groups should form the nucleus of a new family. Evidently the similarity in fatty acid composition of the species in this group supports this idea, and additional comparative studies of lipid composition might be of some value for evaluation of the taxonomic distances among these bacterial groups.

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BINDING OF ENTEROTOXIN FROM *CLOSTRIDIUM PERFRINGENS* TYPE A TO LIVER CELLS IN VIVO AND IN VITRO

The Enterotoxin Causes Membrane Leakage

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Skjelkvåle R, Tolleshaug H & Jarmund T Binding of enterotoxin from *Clostridium perfringens* type A to liver cells *in vivo* and *in vitro*. The enterotoxin causes membrane leakage. Acta path microbiol scand Sect. B 88 95-102 1980

Enterotoxin from *Clostridium perfringens* was shown to retain its biological activity after labelling with ¹²⁵I. When injected intravenously into mice and rats, most of the radioactivity in the organs was present in the form of intact toxin. Studies of the tissue distribution of labelled enterotoxin showed the largest amounts in the liver, where the activity reached a maximum 10-15 min after administration. The highest concentration per g tissue was found in liver and kidneys. The radioactivity was excreted in the urine as a mixture of intact labelled toxin and low molecular weight degradation products. *In vitro* studies with purified parenchymal liver cells showed rapid release of lactate dehydrogenase (LDH) during treatment with enterotoxin, thus indicating severe membrane damage.

Key words: *Clostridium perfringens*; enterotoxin; membrane leakage.

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Clostridium perfringens produces a variety of biologically active metabolites, among which is an enterotoxin associated with outbreaks of food poisoning (23).

In cases of food poisoning, the enterotoxin acts mainly on the intestinal tract and causes diarrhoea. Very little is known about the mechanism by which the enterotoxin causes membrane transport alterations, metabolic disturbances and tissue damage leading to clinical manifestations.

The disturbances in electrolyte and fluid transport due to the action of the enterotoxin have been studied in experimental animals (11, 13). Epithelial squamation has been induced in ileal loops treated with whole cells (4) or highly purified enterotoxin (12, 14). The lethal nature of the enterotoxin has been established after intravenous or intraperitoneal injection resulting in death of experimental animals

with hyperaemic small intestinal mucosa and congestion in liver, lungs, spleen and kidneys (16). No information is available about the distribution and binding of the toxin in the tissues. After episodes of food poisoning, a relatively high level of enterotoxin has been found in faeces and a significant increase in anti-enterotoxin titre in human serum has been demonstrated (21). Similar data have been obtained from human volunteers after ingestion of purified enterotoxin (22). It is not known whether this is the result of a local immune response or a general immune response due to absorption of the enterotoxin.

The present report describes the plasma clearance, distribution and excretion of purified ¹²⁵I-labelled enterotoxin after intravenous injection into mice and rats. Experiments showing the effect of the toxin on the membranes of isolated rat hepatocytes are also included.

MATERIALS AND METHODS

Experimental Animals

The majority of the experiments were performed on white male mice (NMRI/BOVI) weighing 22–25 g while white male rats (MÖLL/WISTAR) weighing 130–150 g were used in some experiments. All experimental animals were received from The Animal Department National Institute of Public Health Oslo.

Enterotoxin

Clostridium perfringens type A strain NCTC 8239 was used for the production of enterotoxin in Duncan and Strong (DS) sporulation medium (3). The enterotoxin was purified by a modification (5) of the method described by *Salaguchi et al* (19).

Iodination of Enterotoxin

Purified enterotoxin was labelled with ^{125}I using a modification of the chloramine T method (6) as described elsewhere (20). The following reagents were added to a 3 ml glass tube during continuous mixing: (1) 10 μl carrier free $\text{Na } ^{125}\text{I}$ (500 μCi) in 0.1 M NaOH; (2) 10 μl 0.5 M phosphate buffer pH 7.0; (3) 10 μl toxin (1 mg/ml); (4) 10 μl chloramine T (1.2 mg/ml). The reaction was terminated after 40 s by the addition of 25 μg sodium metabisulphite dissolved in 300 μl 0.1 M phosphate buffer pH 7.0. The iodination was performed at room temperature. The labelled protein fraction was separated from the unreacted iodide by gel filtration of the reaction mixture through a Sephadex G 50 column (0.9 \times 15 cm) equilibrated in phosphate buffered saline (PBS) pH 7.5 (0.1 M NaCl, 0.05 M phosphate) containing 0.2% bovine serum albumin (BSA) and 0.02% sodium azide. Eluted fractions of labelled protein were combined and incorporation of ^{125}I was calculated. This procedure yielded a labelled preparation containing approximately 0.5 atoms of ^{125}I per molecule of enterotoxin. When tested in the guinea pig skin test (25) the labelled enterotoxin was found to be biologically active. An identical quantity of BSA was labelled by the same procedure except that the concentration of chloramine T was doubled. Labelled BSA was prepared for use in control studies. Labelled preparations were stored at -20°C .

Isotopic Clearance of Enterotoxin and Localization of Enterotoxin in Tissues

^{125}I labelled enterotoxin was injected intravenously into one of the tail veins. In order to follow the clearance of toxin from blood and the distribution of toxin in tissues the animals were decapitated after ether anaesthesia at different intervals following injection.

For determination of total radioactivity the organs were removed, weighed and homogenized in 0.15 M KOH and the total radioactivity was measured.

In preliminary experiments in which attempts were made to identify the labelled substances in the tissues the various organs were homogenized in PBS. One ml of the homogenate was precipitated with 2 ml 30% trichloroacetic acid (TCA) and left at 20°C for 1 h. The suspension was centrifuged, the pellet washed with 5% TCA and the radioactivity in the pellet and supernatants measured.

Other samples of the homogenate were centrifuged 5 min at $3000 \times g$ to remove the red supernatant was subjected to sodium dodecyl sulphate (SDS)-acrylamide gel electrophoresis in glass tubes ($5 \times 5 \text{ cm}$) as described below. After electrophoresis gels were sliced and the radioactivity in each piece measured.

Stability of ^{125}I Labelled Enterotoxin in Blood

In vitro stability of labelled enterotoxin in blood was studied by adding ^{125}I labelled toxin to heparinized blood (200 μg toxin/ml). The mixture was incubated at 37°C for 10, 30 and 60 min. Following incubation whole blood was centrifuged to produce plasma. Pellets were washed three times with PBS and radioactivity in supernatants and pellets was measured.

One ml samples of the plasma were treated with an equal volume of 30% TCA. The supernatant was separated and counted and the precipitate washed three times with 5% TCA. Determination was made of radioactivity of each wash as well as that of the precipitate.

Disc Gel Electrophoresis

Disc gel electrophoresis was performed according to *Davies* (2) on 7% acrylamide gels with tris (bis) (2)-aminomethane glycine pH 8.5 as the buffer. Sodium dodecyl sulphate (SDS)-acrylamide electrophoresis was performed as described by *Osborn* (27). After electrophoresis the gels were sliced and the radioactivity in each piece was measured.

Binding Properties

The antibody binding properties of ^{125}I enterotoxin were tested in a solid phase system. Whole antiserum was used to coat polystyrene test tubes (8). Conditions for incubation of antiserum and buffers were as described previously.

Preparation of Purified Hepatocytes

Rat liver cells were prepared by a modification (26) of the collagenase perfusion method of *Friend* (1). Parenchymal and non parenchymal cells were separated by differential centrifugation (1). 95–99% of the hepatocytes excluded trypan blue, purified hepatocytes were suspended in an incubation medium containing essential salts and defatted BSA (26).

In vitro Test for Membrane Damage

Different concentrations of enterotoxin were added to suspensions of hepatocytes in the minimal medium (10% cells/ml) and incubated in a shaking water bath at 37°C . Portions of the cell suspensions were removed at different intervals and the hepatocytes were separated from the medium by centrifugation through phthalate ($d = 1.05$) as described previously (1). The supernatant was tested for lactate dehydrogenase activity below and LDH activity was used as a measure of membrane damage.

mical Determinations
 lactate dehydrogenase (LDH) (L lactate NAD oxidoreductase EC 1.1.1.27) was assayed in a reaction

protein was determined according to the method of
 7 *et al* (10) with BSA as standard

ing Equipment
 radioactivity measurements were made in an LKB
 1280 automatic gamma counter

RESULTS

LD₅₀ for purified enterotoxin in mice is
 approximately 2 µg (19-24). After intravenous
 administration of enterotoxin in the relevant dose
 per minute amounts of toxin are present in the
 blood and these can be determined only by the use
 of labelled preparations

Stability of ¹²⁵I labelled Enterotoxin in Blood

¹²⁵I labelled enterotoxin was incubated with
 uncoagulated whole blood for different times and the
 stability and binding to blood cells were calculated
 (Table 1). There was no increase in non TCA
 precipitable radioactivity (later referred to as free
 toxin) *in vitro* during the incubation period. Some 6-
 7% is bound to the blood cells and 92% of the
 injected ¹²⁵I labelled enterotoxin was found as TCA
 precipitable material.

(Fig. 1) When blood samples were taken 20 s
 after injection of toxin into a tail vein approxima-
 tely 65% of the total injected dose could be
 counted for in the whole blood, if one assumes the
 blood volume of the mice to be 7% of the body

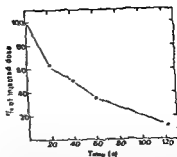


Fig. 1 Clearance curve of ¹²⁵I labelled enterotoxin from
C. perfringens (37 µCi/µg) after intravenous injection
 into mice (100 ng per mouse). Each point represents the
 average of four measurements

weight. After 120 s the amount of labelled toxin in
 the blood was reduced to approximately 10% of the
 injected dose. Results from the study of radioacti-
 vity in tissues obtained at various times after
 challenge indicate that large amounts of the labelled
 enterotoxin were already taken up by liver and
 kidneys.

Although the blood accounts for a minor part of
 the total radioactivity because of the rapid removal
 of toxin from the circulation interesting changes
 take place in blood radioactivity during the test
 period. The amount of free toxin
 and TCA precipitable
 material were reduced to 78% and 66% respectively

Organ Distribution of ¹²⁵I labelled Enterotoxin

In order to ascertain whether the total radioacti-
 vity present in the tissues could be used as a
 measure of the presence of the toxin it was
 necessary to determine to what extent the radioacti-
 vity in the organs was present as TCA precipitable
 material presumably as intact toxin.

Groups of 2-3 mice were used

TABLE 1 *In vitro* Stability of ¹²⁵I labelled Enterotoxin from *C. perfringens* in Heparinized Rat Blood at 37 °C

Incubation time	Free ¹²⁵ I	TCA precipitable radioactivity ¹	Radioactivity bound to blood cells
10 min	7%	92%	6%
30 min	1%	92%	6%
60 min	1%	92%	7%

¹ ¹²⁵I labelled enterotoxin (37 µCi/µg) was incubated at 37 °C with whole blood (200 ng toxin/ml blood)

² Non precipitable radioactivity referred to as free ¹²⁵I

³ Trichloroacetic acid precipitable material

TABLE 2 Radioactivity in Tissues after Intravenous Injection of ^{125}I labelled Enterotoxin from *C. perfringens*

Organ	Time after injection ^{a)} (min)	% radioactivity in precipitate ^{b)}	% radioactivity as intact toxin
Liver	10	98	97
	30	93	94
	60	91	
Kidney ^{c)}	10	91	87
	30	89	87
	60	90	
Lungs	10	87	71
	30	77	60
	60	69	
Spleen	10	79	
	30	79	
	60		
Heart	10	70	
	30	79	
	60		
Intestines	10	91	87
	30	85	83
	60	83	

a) The animals were injected with 400 ng of ^{125}I labelled enterotoxin (37 $\mu\text{Ci}/\mu\text{g}$)

b) Trichloroacetic acid precipitable material

c) As assayed by SDS gel electrophoresis

No observation made

the tissues were processed as described in Materials and Methods. The major part of the radioactivity in the tissues tested was present in TCA precipitable form (Table 2). Analysis by SDS polyacrylamide gel electrophoresis showed that the radioactive material moved in the gel at the same rate as intact toxin. From these data it would seem justified to use the distribution of the total radioactivity in the tissues as a measure of intact ^{125}I labelled toxin.

The results of typical experiments showing the distribution of radioactivity in the tissues after intravenous administration of labelled enterotoxin to mice and rats are given in Fig. 2. Groups of animals were injected each group with different amounts of toxin and the data were expressed as percentages of the injected dose of radioactivity recovered per organ.

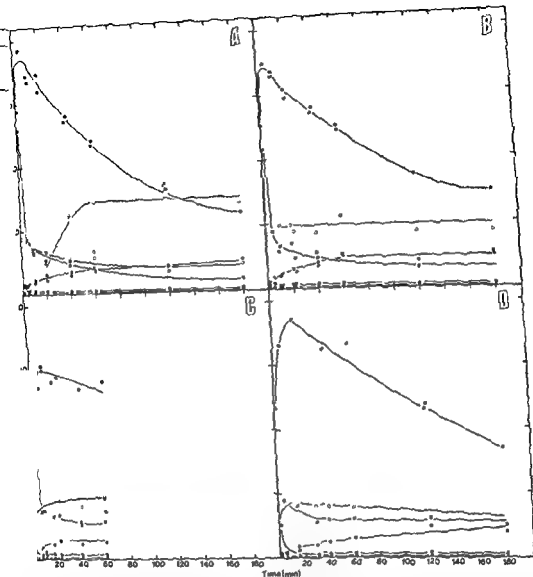
It can be seen (Fig. 2 A) that after intravenous injection of 100 ng ^{125}I labelled enterotoxin into mice, the largest amount of radioactivity was found in the liver where it increased rapidly to reach a maximum about 10–15 min after administration and then declined slowly.

The uptake of ^{125}I labelled enterotoxin by the

kidneys showed a similar pattern. Lower radioactivity were found also in lungs and intestines (including contents), spleen and skin. Radioactivity was low and is not shown in Fig. 2.

The results illustrated in Fig. 2 A were obtained after injection of very small amounts of toxin (100 ng/mouse). Results from experiments where mice were injected with much higher doses of toxin (100 ng ^{125}I labelled + 1000 ng unlabelled) showed that the distribution in tissues and elimination from the organs were essentially unchanged (Fig. 2 B). When the injected dose of toxin increased to the LD_{50} value (100 ng ^{125}I labelled + 2000 ng unlabelled) the tissue distribution was similar (Fig. 2 C) but the elimination from the blood was dramatically reduced. This is probably due to reduced circulation. The mice developed symptoms shortly after injection and none survived more than 60 min. It may be concluded from these results that the tissue distribution and clearance rate from the blood of intravenously administered enterotoxin were affected by the injected dose.

The tissue distribution in rats is almost identical with that described in mice (Fig. 2 D).



2 Distribution of ^{125}I labelled enterotoxin from *C. perfringens* (37 $\mu\text{Ci}/\mu\text{g}$) in various organs after intravenous injection into mice (A, B and C) and rats (D).

Groups of animals were injected with identical amounts of enterotoxin and killed and bled at different periods of time.

The organs were removed and processed as described in Materials and Methods. The data are plotted as percentage of the total injected radioactivity recovered per organ. Each point represents one animal.

100 ng ^{125}I labelled enterotoxin per mouse

100 ng ^{125}I labelled enterotoxin + 1,000 ng unlabelled enterotoxin per mouse

100 ng ^{125}I labelled enterotoxin + 2,000 ng unlabelled enterotoxin per mouse

800 ng ^{125}I labelled enterotoxin per rat

liver

kidneys

■ blood

☆ intestines

● lungs

▲ heart

□ spleen

○ urine

When the data in Fig. 2A were expressed as percentages of the injected dose of radioactivity per g of tissue (Fig. 3), the labelled enterotoxin shows a preference for the liver and kidneys with the highest affinity to liver tissue.

After intraperitoneal injection of labelled enterotoxin the uptake in the organs occurred more slowly, and in most cases the radioactivity was lower than after intravenous injection (data not shown).

For comparison a group of mice was injected intravenously with 100 ng ^{125}I labelled BSA (specific

activities demonstrated are primarily due to membrane damage but the rapid release of LDH from hepatocytes during treatment with enterotoxin would support this hypothesis

Recently McDonel & McClane (15) demonstrated that Vero (African green monkey kidney) cells had binding sites for *C. perfringens* enterotoxin. The cells responded with morphological alteration, inhibition of macromolecular synthesis and increased permeability. Our results are in agreement with these findings.

The chain of events which takes place after binding of enterotoxin to the cell membrane is at present unknown. Preliminary studies with subcellular distribution of cell-associated enterotoxin in hepatocytes indicate that the enterotoxin exerts its effect primarily on the cell membrane. In order to reach a final conclusion as to whether the effect on macromolecular synthesis in mammalian cells is a primary effect of internalized toxin or a secondary effect due to membrane damage, experiments must be performed in cell-free systems.

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EFFECTS OF SOME ANTIBACTERIAL AGENTS ON THE PHAGOCYTOSIS OF ³²P-LABELLED *ESCHERICHIA COLI* BY HUMAN POLYMORPHONUCLEAR CELLS

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Melby K. & Midtvedt T. Effects of some antibacterial agents on the phagocytosis of ³²P-labelled *Escherichia coli* by human polymorphonuclear cells. Acta path. microbiol. scand. Sect. B 89: 103-106, 1980.

Gentamicin, trimethoprim, cephalothin, colistin, erythromycin, oxytetracycline and chloramphenicol were studied in a phagocytic system. A radiolabelled strain of *E. coli* was used as test bacterium and human polymorphonuclear cells were used as phagocytes. Except for trimethoprim and cephalothin, there was a tendency towards depression of the process of phagocytosis in the presence of high concentrations of the various antibiotics. Colistin in a high concentration (83 µg/ml) exerted the most significant effect.

* Key words: Phagocytosis, antibiotics, human polymorphonuclear cells, *E. coli*.

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8 vii 79 Accepted 8 xi 79

It is well known that during an attack by an agent, antibacterial drugs only assist the immune system in eradicating the invading

A possible interaction between the cells of the phagocytic system and these drugs has received increased attention (1, 5, 7, 8, 9, 10). The present report is an attempt to evaluate the effects of some antibacterial drugs on the process of phagocytosis of a radiolabelled strain of *Escherichia coli* by human polymorphonuclear cells (PMN).

MATERIALS AND METHODS

Human polymorphonuclear cells (PMN) were isolated (4) from whole blood (5 IU/ml) obtained from donors by venipuncture and thereafter transfered to glass tubes in order to yield a monolayer of cells of which approximately 80% were PMN as described previously (14).

Cells obtained from healthy blood donors were washed and stored in small aliquots at -80 °C. The strain used has been described earlier as

a member of the species *E. coli* according to Bergey's Manual (3). The strain was stored in lyophilized state at -5 °C and also kept in subculture on lactose bromthymol blue agar plates.

Culture technique. The medium used was prepared according to Benacerraf *et al.* (2). The bacterium was cultivated in 50 ml medium for 18 h. Four ml of this culture was transferred to 200 ml of medium to which was added 1 mCi ³²P-labelled orthophosphate. The bacterial growth was discontinued after 200 minutes by rapid cooling of the suspension to 0 °C. The number of viable bacteria determined as colony forming units (CFU) after dilution and plating was 10⁹ ± 0.5 log. All incubations were kept aerobically in a shaker at 37 °C.

Phagocytosis assay (USA). The bacterial suspension was washed three times with a chilled (4 °C) Krebs-Ringer phosphate buffer which was enriched with 10 mM of glucose.

Phagocytosis assay (USA). After 30 minutes the medium was decanted and a solution of the antibiotic in KRG was added. Half an hour later the

medium was decanted again and the monolayers washed twice with prewarmed KRG (37 °C). Samples of 2.5 ml of the bacterial suspension containing $10^9 \pm 0.5$ log radiolabelled *E. coli* per ml were added and the tubes were left for incubation for 15 minutes at 37 °C. The two types of bacterial suspensions used were bacteria suspended in KRG alone and bacteria suspended in KRG containing 10% normal human serum. Serum was added immediately before the transfer of the bacterial suspension to the test tubes. On termination of the ingestion phase the suspension was decanted and the tubes washed four times with chilled (4 °C) KRG and left for drying. In order to dissolve the cells 2 ml of Lowry's Alkaline Copper Solution (LACS) (11) was added when the tubes were dry. Thereafter samples of 0.2 ml were removed for determination of radioactivity and 0.5 ml for determination of cell protein content. The count per mg cell protein reflects the ingestion of radiolabelled *E. coli*. The effects of the various drugs were expressed by relating counts per mg cell protein in the untreated tubes to the data obtained from tubes incubated with the various drugs. KRG and serum control tubes were always included and all experiments were performed in triplicate.

Determination of protein content was carried out according to the method of Lowry *et al.* (11) modified by Oyama & Eagle (18). Lyophilized human albumin with 96% purity (Sigma Chemical Chemical Comp. Co. USA) dissolved in distilled water was used as reference.

Determination of radioactivity was performed with aliquots in a Hewlett Packard liquid scintillation counter.

Drugs. The antibiotics used and the concentrations applied are shown in Table 1. All drugs were primarily dissolved in distilled water and diluted to the appropriate concentrations using KRG with the exception of oxytetracycline which was dissolved in distilled water and diluted in KRG devoided of Ca^{++} and Mg^{++} . In the presence of oxytetracycline KRG lacking Ca^{++} and Mg^{++} was always used. Trimethoprim lactate was dissolved in distilled water to which HCl (4N) was added to give pH 4.0. For complete dissolution the suspension was kept at 37 °C for 15 minutes. Further dilutions were carried out with prewarmed (37 °C) KRG. In the various concentrations to be tested the pH was neutral.

Statistical analysis. The Wilcoxon White rank test (12) was used ($N = 2$, $P < 0.05$).

RESULTS

The effect of seven antibacterial agents on phagocytosis is shown in Table 1. These results indicate that apparently some have an influence on the ingestion phase of phagocytosis when the PMN monolayer has been pretreated with the various antibacterial drugs for 30 minutes before the start of the ingestion period.

TABLE 1 Influence of Some Antibacterials on Phagocytosis of ^{32}P labelled *E. coli* by Human Polymorphonuclear Cells in vitro

Drug	Concentration $\mu\text{g/ml}$	Per cent of KRG	%
Cephalothin	30	108	a
	300	126	a
Chloramphenicol	10	91	a
	200	99	a
Colistin	8.3	100	a
	83	81	b
Erythromycin	10	93	b
	100	108	a
Gentamicin	5	111	a
	100	114	a
Oxytetracycline	10	93	b
	100	112	a
Trimethoprim	10	106	a
	100	104	a

The results are given as percentages compared with untreated controls and are the arithmetic mean of observations for each drug.

a - $p = 0.1$ b - $p < 0.05$ c - $p < 0.01$

Using high doses of either chloramphenicol or erythromycin in the presence of normal serum during the uptake of bacteria a trend towards depression of the phagocytic process was seen.

Using high concentrations of colistin the relative capacity was reduced to 81% when only serum was present during the ingestion phase ($p < 0.05$) and 76% when serum was present during the period of ingestion ($p < 0.01$).

Gentamicin tested with normal serum present during the period of ingestion seemed to impede the ingestion phase slightly. Using 5 $\mu\text{g/ml}$ as concentration the ingestion was reduced to 81% ($p = 0.1$) whereas when the concentration increased to 100 $\mu\text{g/ml}$ the uptake of bacteria was 114% of the normal uptake ($p = 0.1$).

Oxytetracycline in a dose of 100 $\mu\text{g/ml}$ with normal serum exerted some influence on the uptake of bacteria since only 76% of the normal ingestive capacity was recorded ($p = 0.1$). The difference may be regarded only as a tender influence on the process of phagocytosis.

Apparently trimethoprim and cephalothin had no influence on the process of phagocytosis.

DISCUSSION

accordance with other reports (5, 9, 13, 17) on interaction between beta lactam antibiotics and phagocytosis, no significant effect was shown when using cephalothin.

Colistin, a polypeptide antibiotic very similar to polymyxin B, interferes with the cell membrane function as a transport barrier of smaller molecules. The influence of this antibiotic has been demonstrated that the cell membrane of bacteria becomes leaky. The effect of this antibiotic has been studied on human leucocytes with no measurable effect when using 115 µg/ml (8). Other workers using guinea pig leucocytes and 1200 µg/platelet about a 22% reduction in the ingestive capacity (5). In our experimental system a depressive effect was found with a test concentration of 83 µg/ml. The effect seemed to be most pronounced in normal serum was present during the period of ingestion. This is partly in accordance with other results (13) using rat PMN and the same concentration of colistin. In those studies a depressive effect was found when 83 µg/ml was used. The depressive effect was eliminated by the addition of serum.

The reason for this discrepancy is not clear, it may be due to different affinities of the drug to various types of cells and sera. Thus, high doses of colistin seem to impair the process of ingestion and phagocytosis as shown in our study. Other studies have shown that high concentrations of chloramphenicol inhibit the process of phagocytosis (6, 10). Using 200 µg/ml of this antibiotic it has previously been demonstrated that

of leucocytes containing microorganisms could be demonstrated with either of these antibiotics in the range of 2.5–25 µg/ml. Other studies (8, 9) do not confirm these findings even with high concentrations of tetracycline (426 µg/ml). The results presented in this study support the opinion (1, 7, 13, 15) that the tetracyclines apparently have an inhibitory influence on the process of phagocytosis. However, the concentrations necessary to exert such influence seem to be rather high, though not higher than what can be obtained in the regions of the human body where the drugs are concentrated (6, 16). Our findings support the results obtained from studies on rat PMN (13).

To our knowledge there is no other report dealing with the interaction of trimethoprim and the phagocytic system except our previous studies using rat PMN (13). This compound seems to have no influence on the ingestion phase of the phagocytic process.

It is likely that some antibiotics do have an influence on the process of ingestion when a microbe is to be phagocytosed by PMN. The concentrations apparently necessary to reveal these effects are obtainable in the human body (16). The clinical implications of these findings are difficult to establish. The reduced effect on the uptake of bacteria shown with high doses of colistin is worthy of attention.

It may be concluded that except for trimethoprim and cephalothin there is a tendency towards a depression of the ingestion phase when high concentrations of the other antibiotics tested are applied.

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Results presented also indicate inhibitory effects on the phagocytic process by gentamicin. This confirms previous findings (9, 13).

Very few reports are available regarding the effect of tetracyclines on the process of phagocytosis. In a study by Berg et al. (11) have demonstrated that administration of 12 mg per day in drinking water for four days decreased the efficiency of the phagocytosis of rats to clear carbon particles injected into systemic circulation. Munro & Geisler (15) reported inhibition of the engulfment of a microorganism by the action of chlortetracycline applied to human leucocytes tested in an *in vitro* system. The lowest effective concentration found was 0.01 µg/ml. Forsgren et al. (7) have extended these findings by their studies on *E. coli* and yeast presented to human PMN together with tetracycline or tetracycline. A decreased percentage

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EXPERIMENTAL SALPINGITIS IN GRIVET MONKEYS BY *CHLAMYDIA TRACHOMATIS*

Modes of Spread of Infection to the Fallopian Tubes

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Möller B R & Mårdh P A Experimental salpingitis in grivet monkeys by *Chlamydia trachomatis*
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Experimental infection with *Chlamydia trachomatis* immunotype K was carried out in six grivet monkeys. Three routes of infection were used (A) into the uterine cavity through the cervical canal (B) into the uterine cavity but after ligation of the tubal isthmus followed by curettage of the endometrium (C) into the cervical epithelium. Two monkeys were used for each type of experiment. All monkeys inoculated by routes (A) and (C) developed marked signs of inflammation in the Fallopian tubes. Characteristically the tubes were reddened and swollen. Exudate appeared in the abdominal cavity and in the cul-de-sac. In three of the four monkeys studied a yellowish discharge was seen in the posterior vaginal fornix. An exudate composed mostly of lymphocytes and clusters of desquamated cells was found in the lumen of the tubes. The epithelium was injured in some areas and there were adhesions between the tubes. In the two monkeys tubal occlusion was present two months after infection. A significant rise in antibodies to *C. trachomatis* was seen in the upper genital tract in all monkeys. One of the animals died day 14 post inoculation; autopsy showed peritonitis and perihepatitis. One monkey was inoculated with non-infected yolk sac specimen by route (A) and another by route (B). Practically no inflammatory changes in the genital organs or elsewhere could be detected in these monkeys. The results indicate that *C. trachomatis* can cause salpingitis in female grivet monkeys by canalicular spread from the lower genital tract. The histological findings and the infection route in this experimentally induced salpingitis in the grivet monkeys closely resemble those ascribed to gonococcal salpingitis in man.

Key words: *Chlamydia trachomatis*, salpingitis, experimental infection.

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Chlamydia trachomatis is a common cause of sexually transmitted diseases (8, 9, 12). In the female the organism may produce cervical infection associated with abnormal Papanicolaou smears (1). In the cervix, chlamydiae may reach the tubal lumen and cause acute salpingitis (6). However, mode of spread of the infection from the cervix to the tubes is not known.

In a previous study (11) *C. trachomatis* was introduced into the uterine cavity or into the tubes or into

the uterine cavity of grivet monkeys. Within a few days these monkeys developed salpingitis. However, this study did not confirm the route of infection to the tubes, neither did it simulate conditions comparable to those of naturally occurring infections in the female.

Experiments in grivet monkeys were therefore designed to mimic conditions that might occur in natural infections with chlamydiae in the female, viz. the opportunity for a canalicular spread of such organisms through the uterine cavity to the lumen

of the Fallopian tubes and/or spread via lymphatics and blood vessels through lesions in the cervical epithelium or in the uterine endometrium. The results of these studies are given in the present communication.

MATERIAL AND METHODS

Organism A strain of *C. trachomatis* immunotype K was isolated from the Fallopian tubes of a patient with acute salpingitis. This strain was used to infect a grivet monkey into the uterine tubes as described previously (11). This monkey developed acute salpingitis. In the present study the strain isolated from the patient (No. 771) and that (No. 5155) reisolated from the cervix of the infected monkey were used. The strains had been isolated on cycloheximide treated McCoy cells (10) and passed twice in the yolk sac of embryonated hen eggs. Suspensions of the yolk sac material containing 2×10^5 inclusion forming units per ml were stored at -80°C until use. A hundred fold dilution of the suspension in phosphate buffered saline pH 7.2 was used to inoculate the monkeys.

A yolk sac suspension from a non infected embryonated hen egg diluted in PBS as described above was also tested.

Monkeys Eight female grivet monkeys (*Cercopithecus aethiops*) were used in the experiment. They weighed 2.0–2.6 kg. They had been captured in East Africa and before use had been kept in quarantine for at least six weeks. They were examined and caged as described elsewhere (5).

Specimens were collected on three consecutive occasions from the throat, the urethra, the cervix and the rectum and cultured for *C. trachomatis* mycoplasmas and ureaplasmas. The sampling and culture techniques used were those described elsewhere (11). Blood samples were taken by puncture of the femoral artery in the inguinal region. All samples were collected when the monkeys were anaesthetized using ketamine chloride (Ketalar® 5 mg/kg i.m.).

Experimental infections The monkeys were infected in three different ways.

(A) In monkeys I and II 0.4 ml of the suspension of chlamydiae (strain No. 771) was injected into the uterine cavity using a steel catheter 0.4 mm in diameter introduced through the cervical canal.

(B) In monkeys III and VI the isthmus of the Fallopian tubes was closed by ligatures during laparotomy performed as described elsewhere (5). In these latter monkeys 0.4 ml of chlamydial suspension (strain No. 771) was injected as described in experiment (A). The cervix was then dilated to 1.5 mm using modified

C. culture of the uterine endometrium
1.2 mm
sension of

sac specimen in the same way as in experiment VIII was infected as in experiment (8).

Assessment of lesions and collection of samples Laparotomy was performed on days 4, 7, 21, 31 post inoculation (p.i.). At each laparotomy biopsies taken from the Fallopian tubes and parametria; smears collected by a swab were taken from the sites: the tubal fimbriae, the cul-de-sac and the

Culture studies Cultures for *C. trachomatis* and mycoplasmas (5) were performed in described media. Bacterial cultures were made in brain heart broth (Difco) and on blood haematin and bromthymol blue agar.

Serological studies Surveys for serum anti *C. trachomatis* were carried out by a microimmuno-rescense test using pools of antigen (13). The sera were titrated in two fold dilutions. The lowest dilution was 1:8. All sera were tested for both IgG antibodies to the organism.

Histological studies Tissue biopsies were fixed in 10% formalin and paraffin embedded for histology studies using staining with haematoxylin and

RESULTS

General condition of the infected monkeys Eight monkeys, only one presented any clinical signs of disease. Thus monkey III developed fever, showed slow and prudent movements and loss of appetite. The illness progressed and the monkey died day 14 p.i. The monkey was incapable of eating and drinking for 3 days previously.

Gross lesions In monkeys I, II, V and VI inflammatory lesions were practically identical on days 7, 14 and 21 p.i.; the tubes were red, swollen. Exudate appeared in the abdominal cavity, the tubes and a watery exudate was found in the cul-de-sac. Day 35 p.i. the inflammatory reaction had regressed. On day 60 p.i. the appearance of the tubes was virtually normal. No gross lesions were seen in the parametria, the ovaries and the peritoneal organs including the spleen. No macroscopical signs of inflammation.

Monkey III presented no gross lesions on day 7 p.i. in the tubes, the parametria, the spleen. There was no exudate in the cul-de-sac or in the cul-de-sac. The monkey died on day 14 p.i. Autopsy showed peritonitis and perihepatitis with adhesion of the spleen and the anterior surface of the intestines. The spleen was enlarged. The internal organs and the intestine were normal. There were no signs of pneumonia.

Monkey VII was inoculated with 0.4 ml of

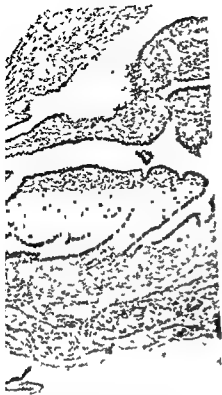


Fig. 1. Histological section of the Fallopian tube of monkey II inoculated into the uterine cavity with *C. trachomatis* immunotype K, day 4 post inoculation. A pronounced inflammatory infiltrate in the tubal epithelium and in the subepithelial tissue. HE. $\times 50$.

IV and in the control monkeys (VII and VIII) throughout the experimental period.

A yellowish discharge was seen in the posterior vaginal fornix of monkeys II, V and VI from day 7 and during the following 2 to 3 weeks. There were no erosions of the cervix.

Histological findings. Monkeys I, II, V and VI all presented histological inflammatory changes in the lower genital tract. The alterations were pronounced



B

Fig. 2. Histological sections of the Fallopian tube of monkey VI inoculated into the cervical epithelium with *trachomatis* immunotype K. (A) On day 7 post inoculation pronounced inflammatory infiltrate in the tubal epithelium and in the subepithelial tissue can be seen. The epithelium is injured in some areas. HE. $\times 60$.

(B) On day 14 post inoculation pronounced inflammatory infiltrate in the tubal epithelium and in the subepithelial tissue can be seen. The epithelium is injured in some areas. HE. $\times 60$.





Fig 3 Histological section of the Fallopian tube of monkey V inoculated into the cervical epithelium with *C trachomatis* immunotype K, day 60 post inoculation. Adhesions between the mucosal folds and infiltration of a small number of inflammatory cells in the subepithelial tissue and the muscular layers can be seen. HE $\times 20$.



Fig 4 Histological section of the Fallopian tube of monkey VI, inoculated into the cervical epithelium with *C trachomatis* immunotype K, day 60 post inoculation. The tubal epithelium is atrophic and flattened, the lumen is diminished and the muscular layers more thickened. HE $\times 15$.

in monkeys II, V and VI, but less so in monkey I. In monkey I, the tubal epithelium and the subepithelial tissue showed infiltration with lymphocytes and some polymorphonuclear leukocytes on days 7, 14 and 21 p.i. The tubal epithelium was intact and there was no inflammation in the tubal muscular layers in the subserosa or in the parametria. On days 35 and 60 p.i., practically no histological signs of inflammation were found.

In monkeys II, V and VI, there was infiltration in the tubal epithelium and the subepithelial tissue with abundant lymphocytes and some leukocytes on day 4 p.i. (Fig. 1). On days 7, 14 and 21 p.i., the lumen of the Fallopian tubes contained an exudate with lymphocytes and clusters of desquamated cells. The epithelium was injured in some areas and a few adhesions were seen between the mucosal folds (Figs. 2A and B). On day 35 p.i., a moderate inflammatory reaction was found in the epithelium and in the subepithelial tissue, but the epithelial

lining of the tubes was practically intact. Adhesions between the mucosal folds were present in monkeys V and VI. On day 60 p.i., the tubes of monkeys V and VI were normal. In monkey V, the mucosal folds thickened and adhesions were still present, but a small number of inflammatory cells were seen in the subepithelial tissue. In monkey VI, the lumen of the tubes was diminished and the tubal epithelium atrophic and flattened. The muscular layers increased and inflammatory cells were present in the subepithelial tissue. The parametria revealed only minimal inflammatory infiltration, mostly in the subserosa (Figs. 3 and 4).

Monkey III presented no signs of inflammation in the tubes or parametria on days 4 and 14. Autopsy on day 14 p.i. showed slight inflammatory infiltration of the subserosa of the Fallopian tubes, while the muscular layers, the subepithelial tissue and the epithelium were normal. Moderate infiltration of inflammatory cells was found in the



Fig. 1. Histological section of the parametria of monkey I, inoculated into the uterine cavity after 1 day of the infection with *C. trachomatis* immunotype K. Day 4 post-inoculation. Moderate infiltration of inflammatory cells, particularly around the blood vessels can be seen. E. = 120.

parametria, particularly around the blood vessels. There was no inflammation in histological sections of the portio, the cervical canal, the uterus or ovaries. Sections from the liver showed

swollen and a great number of polymorphonuclear leukocytes and plasma cells were seen. No signs of infection were found.

Monkey IV, on day 4 post-inoculation, slight inflammatory infiltration was found in the parametria but no oedema. On day 7 post-inoculation a moderate

inflammation was found in the parametria.

though very slight, were confined to the subserosa of the Fallopian tubes and the parametria.

Haematological findings. Monkeys III and IV showed a pronounced rise in the erythrocyte sedimentation rate (ESR) during the first week post-inoculation, viz. from 3 to 52 mm/h and from 2 to 60 mm/h respectively. In the same monkeys there was a marked rise in the leucocyte count (LC), viz. from 8 100 to 17 300/ μ l and from 6 700 to 10 600/ μ l. The other six monkeys studied showed no or only slight changes in the ESR and the LC after the infection (Table 1).

Isolation of *C. trachomatis*. Chlamydiae could not be recovered from monkey I during the investigation period. In monkey II the organism was isolated from the tubes and from the cul-de-sac on day 21 post-inoculation. In monkey III *C. trachomatis* was isolated from



Fig. 2. Histological section of the liver of monkey III, inoculated into the uterine cavity after 1 day of the infection with *C. trachomatis* immunotype K. Day 4 post-inoculation. Moderate inflammation can be seen. E. = 120.

TABLE 1 *Erythrocyte Sedimentation Rate (ESR) and Leukocyte Count (LC) × 10³ in Grivet Monkeys Experimentally with C trachomatis Immunity Type A (Monkeys I-VI) and in Two Control Monkeys (VII and VIII)*

		Days post inoculation						
Site of inoculation	Monkey No		0	4	7	14	21	35
Uterus	I	ESR	ND ^a	5	8	4	2	ND
		LC	ND	5.8	5.8	6.6	ND	ND
	II	ESR	2	8	7	1	3	2
		LC	6.9	6.2	8.0	7.2	6.5	9.2
Uterus (followed by curettage)	III	ESR	3	ND	52	ND ^b		
		LC	8.1	ND	17.3	9.0		
	IV	ESR	2	60	50	17	2	1
		LC	ND	6.7	10.6	9.4	9.1	3.9
Cervix	V	ESR	2	2	20	16	2	ND
		LC	8.1	6.3	9.1	6.8	6.5	ND
	VI	ESR	1	2	3	3	2	ND
		LC	5.4	5.8	4.6	4.9	6.4	ND
Controls	VII	ESR	2	2	ND	4	4	ND
		LC	7.5	7.0	ND	6.8	6.6	ND
	VIII	ESR	3	5	4	4	ND	ND
		LC	6.2	6.2	6.8	6.7	ND	ND

^a ND - not done

^b Monkey III died on day 14 p.i.

the cervix on days 7 and 14 p.i. and from the uterine cavity on day 14 p.i. Samples collected from the tubes and the fimbriae revealed no growth of chlamydia nor did samples collected from the surface of the spleen the intestine and from the lungs. In monkeys IV, V and VI chlamydiae were isolated days 14, 21 and 7 p.i. respectively.

From the control monkeys viz monkeys VII and VIII no chlamydiae could be isolated on any of the sampling occasions.

No mycoplasmas were isolated from the specimens of the eight monkeys collected in connection with the laparotomies. With the exception of sparse growth of coagulase positive staphylococci isolated from the fimbriae and cul de sac day 21 p.i. of monkey II and of coagulase negative staphylococci from the tubes of monkey IV day 7 p.i. the bacterial cultures were negative.

Antibody studies. No antibodies to *C. trachomatis* were detected in serum drawn before the inoculations. No IgM or IgG antibodies to *C. trachomatis* were detected during the investigation period in the serum samples from monkeys III (which died day

14 p.i.) and V or from the two control monkeys II and VIII while IgG antibodies were found in monkey IV and VI (Table 2).

DISCUSSION

Salpingitis may be caused by an infection from adjacent organs generally an inflamed duct or as in the vast majority of cases infection ascending from the lower genital tract. In principle two groups of ascending infections are recognized. One type is the so-called gonococcal salpingitis in which the causative organism enters the tubes from the cervix by canalicular spread along the uterine mucosa to the tubes. This type of salpingitis produces a suppurative inflammation of the endosalpinx that may lead to tubal occlusion. The parametria are usually not affected. In the non-gonococcal type of salpingitis the organisms are believed to invade the tissue through lesions of the cervical epithelium or of the uterine

2 Titres of IgM and IgG Antibodies to *C. trachomatis* in Grivet Monkeys Infected Experimentally by the Organism and Control Monkeys (II and III)

Monkey No		Days post inoculation				
		7	14	21	35	60
I	IgM	-	-	-	-	-
	IgG	-	-	-	64	-
II	IgM	-	64	32	-	-
	IgG	-	-	128	512	128
III	IgM	-	-	-	-	-
	IgG	-	-	128	128	64
IV	IgM	-	-	-	-	-
	IgG	-	-	-	-	16
V	IgM	-	-	16	-	-
	IgG	-	-	16	1024	512
VI	IgM	-	-	-	-	-
	IgG	-	-	-	-	-
VII	IgM	-	-	-	-	-
	IgG	-	-	-	-	-
VIII	IgM	-	-	-	-	-
	IgG	-	-	-	-	-

Monkey III died on day 14 p.i.

um and are spread via lymphatics and blood vessels to the tubes parametria and broad ligaments. In this latter type of salpingitis pronounced inflammation occurs in the parametria. The inflammation in the tubes is generally confined to the interserosa and the endosalpinx is usually unaffected.

Besides being caused by *Neisseria gonorrhoeae*

isopathological picture similar to that found in these later monkeys was demonstrated in two patients with pyosalpinx caused by *C. trachomatis* (5).

The present study seems to indicate that chlamydiae may spread canalicularly from the cervix to the upper genital tract. When the isthmus of the tubes was closed by ligatures, as was the case in monkeys II and IV, no inflammation of the tubes or parametria occurred. However, these two monkeys presented a marked rise in the ESR and the LC.

Monkey III died on day 14 p.i. Autopsy of this monkey showed peritonitis and enlargement of the

spleen. Perihepatitis also occurred. Acute fibrinous perihepatitis reported already nearly fifty years ago by Friess, Hugh (4) and Curtis (2) has been described as a complication of gonorrhoea. Recently Muller, Schoop and co-workers (7) reported peritonitis and perihepatitis in young women with high antibody titres to *C. trachomatis*.

Autopsy of monkey III revealed no evidence of a generalized infection although the spread of *C. trachomatis* from the uterine cavity to the abdominal organs probably occurred via blood vessels and lymphatics. Canalicular spread was not possible because of the ligatures.

In 1946 Falk (3) reported on cornual resection in more than 1 000 women with infected Fallopian tubes. The patients were followed up for more than eight years and not one single case of recurrent salpingitis was found. Falk concluded that the cornual resections prevented canalicular spread from the cavum uteri to the tubes and protected against ascending organisms. The author suggested that in most cases of primary salpingitis gonococci are the causative agents and that such organisms may ascend from the lower genital tract through the uterus to the tubes. However, he was only able to recover gonococci from the tubes of these patients in a few cases. Chlamydial infection was not included in that material. In the present study we found that ligation of the tubal isthmus prevented *C. trachomatis* from reaching the tubal epithelium and causing salpingitis. *N. gonorrhoeae* and *C. trachomatis* both induce salpingitis which is very similar in histopathological appearance. Also the method of spread of the organism to the tubes is the same.

Sequelae of salpingitis in the female is known to cause impaired fertility and sterility due to obstruction of the lumen of the tubes. In the present study *C. trachomatis* produced inflammation in the tubes of four monkeys. In two of them (monkeys I and II) the tubal epithelium was intact day 60 p.i. and there were no inflammatory reactions. However, in monkeys V and VI the epithelium of the tubes was still not normalized on day 60 p.i. Thus, in monkey V the mucosal folds were thickened and adhesions between the folds were seen while in monkey VI the lumen was reduced in diameter and the epithelium was atrophic and flattened. Such changes may impede transport of a fertilized ovum from the ovary to the uterus thereby impairing fertility or causing sterility and an increased risk of ectopic pregnancy (14).

The present study showed that in grivet monkeys *C. trachomatis* is able to attack the tubal epithelium and produce salpingitis. The infection may reach the tubes via canalicular spread from the cervix. In some of the monkeys studied partial

TABLE 1 Bacterial Strains Examined

<i>F. necrophorum</i>	VPI 6161 SPH 1 N 167
<i>F. mortiferum</i>	VPI 0473 VPI 4249 VPI 5696
<i>F. gonidiaformans</i>	VPI 0482A VPI 4381 VPI 4879 VPI 11360
<i>F. varium</i>	ATCC 8501 VPI 0499A VPI 4234 Lille 109
<i>F. naviforme</i>	VPI 4877 VPI 11936C VPI 12188
<i>F. russi</i>	VPI 0307 VPI 10646 VPI 11018
<i>F. plauti</i>	VPI 0310 VPI 0311 VPI 0312
<i>F. nucleatum</i>	F1 F3 F5 F6 F7 F9 F10 F11 F14 F18

VPI Virginia Polytechnic Institute and State University Blacksburg Virginia USA
ATCC American Type Culture Collection Rockville Rockville Maryland USA
Lille Institute Pasteur de Lille Lille France
Strains SPH 1 and N 167 were received from Dr Justesen Copenhagen Denmark and Dr Beuren Lille France respectively
The *F. nucleatum* strains were own isolates

MATERIALS AND METHODS

Microorganisms

The bacterial strains examined are listed in Table 1. They were cultivated in an enriched fluid medium based on tryptone (1). After harvesting by centrifugation the cells were washed twice in saline and stored as a paste at -20°C until used.

Isolation of LPS

Frozen cells (1 g) were treated with constant stirring with 45 per cent aqueous phenol (35) (5 ml) at 20°C for 30 min. The LPS were purified from the water phase by ultracentrifugation ($100\,000 \times g$ for 90 min) and by treatment of the pellet with ribonuclease and deoxyribonuclease (8).

Gas Liquid Chromatography (GLC)

For the determination of neutral sugars samples of LPS were hydrolysed in sealed ampoules with 0.1 N HCl for 48 h at 100°C . The hydrolysed samples were neutralized with Amberlite IRA 410 HCO_3^- form and the aldoses converted to alditol acetates (24). GLC was performed by a Perkin Elmer 900 gas chromatograph

USA. The carrier gas (N_2) flow was 30 ml per min and the injector and detector temperatures were 200°C and 250°C respectively whereas the column was programmed from 180 to 240°C at a rate of 2°C per min. D-xylose was used as an internal standard. The gas chromatographic peaks were tentatively identified by comparing retention times with those of standards including a hydrolysate of a *Salmonella typhimurium*

LPS which contains both L and D-*D* mannoheptose (20) (obtained from D. O. L. Freiburg FRG).

For analysis of fatty acid composition LPS (0.5 to 1.5 mg) were methanolyzed in 2 ml methanol (1.5 ml) in teflon lined screw-cap vials at 85°C for 18 h (17). The fatty acid methyl esters were extracted with hexane and treated with methyl anhydride/acetonitrile (14) before GLC on a column packed with 3.8 per cent SE-30 on 60-80 mesh (Serva Heidelberg FRG). The temperature was programmed from 170 to 270°C at a rate of 2°C per min. Otherwise the experimental conditions were as described above. As internal standards heptadecanoic acid was used. A HLD fatty acid mixture (Supelco Inc.) 3 hydroxy tetradecanoic and 3 hydroxy hexadecanoic acid (Applied Science Laboratories Inc. State College Pennsylvania USA) were used as external reference standards.

Paper Chromatography

For identification of amino sugar samples were first hydrolysed with 2 N HCl at 100°C . Acid was removed from the hydrolysates by evaporation *in vacuo* over NaOH pellets. Circular paper chromatography was done using Whatman No. 1 paper solvent system butanol of pyridine/water (6:4:1 v/v). Detection reagents used were ninhydrin and alkaline silver nitrate (28).

Colorimetric Determination of LDO

The presence of LDO was examined by the tannic acid method (33). Samples of LPS were hydrolysed with 0.02 N H_2SO_4 for 20 min at 100°C . The density was examined in the range of 500 to 600 nm. A peak at 550 nm was recorded as a positive result.

RESULTS

LPS were obtained in varying yields from the strains listed in Table 2 and Table 3. The neutral sugars and fatty acids accounted for 10 per cent of the dry weight of the preparations. When different batches of LPS from the same strain were examined the total amount of neutral sugars varied somewhat but the molar ratios of different sugar constituents were similar.

Heptose 1 which had a retention time close to that of L-glycero D-mannoheptose was present in all LPS as a main sugar component (17) particularly so in the LPS isolated from the strains of *F. gonidiaformans*. Galactose was found in LPS preparations examined except those from strains VPI 4381 VPI 4234 and Lille 109. Glucose could not be detected in *F. necrophorum* SPH 1 *F. mortiferum* VPI 5696 and *F. varium* VPI 0499A but was otherwise present.

E 2 Neutral Sugars in LPS from *F. necrophorum*, *F. mortiferum*, *F. gonidiaformans*, *F. varium*, *F. naviforme*, Strain VPI 4877 and *F. russi* Strain VPI 0307

Strain	LPS yield	Neutral sugars (%)	Molar ratios of				
			Heptose I	Heptose II	Glucose	Galactose	Rhamnose
<i>necrophorum</i>							
PI 6161	1.45	10.2	1		tr	1.6	
PI 11	1.31	17.8	1			2.5	
PI 167	2.53	11.5	1		0.3	1.3	
<i>mortiferum</i>							
PI 0473	2.16	22.3	1	0.3	0.4	0.5	
PI 4249	6.16	25.9	1	0.4	0.4	0.3	
PI 5696	5.48	10.5	1			1.0	0.8
<i>gonidiaformans</i>							
PI 0482A	1.75	18.8	1	0.1	0.3	0.2	
PI 4381	1.72	22.6	1	0.1	tr		
PI 4879	1.66	14.4	1	0.1	tr	0.1	
PI 11360	1.00	18.1	1	0.1	0.2	0.2	
<i>varium</i>							
ATCC 8501	3.50	7.6	1	0.1	0.8	1.0	
VPI 0499A	3.53	20.3	1			0.7	
VPI 4234	3.27	31.9	1	0.7	0.6		
ATCC 109	0.82	14.5	1	0.8	0.8		
<i>naviforme</i>							
VPI 4377	1.73	25.2	1		0.5	0.2	
<i>russi</i>							
VPI 0307	1.18	11.8	1		0.4	1.0	0.9

All freeze-dried LPS obtained from 1 g of wet bacteria

(trace amounts)

Heptose I : retention time GLC similar to L-glycero-D-mannoheptose

Heptose II : retention time GLC similar to D-glycero-D-mannoheptose

Paper chromatography revealed the presence in all LPS of glucosamine. Galactosamine was not detected. Most preparations contained nohydramine components with a faster chromatographic mobility than glucosamine. As these compounds were silver nitrate negative (not reducing) their structure was not examined.

Small amounts (less than 1 per cent) of KDO were present in all LPS.

All LPS preparations examined contained α -tetradecanoic acid and 3-hydroxy-tetradecanoic acid (Table 3). The LPS isolated from strains of *F. necrophorum* contained in addition α -hexadecanoic and 3-hydroxy-hexadecanoic acid. The former constituent was also present in LPS prepared from *F.*

necrophorum SPH 1, *F. russi* VPI 0307 and the strains of *F. mortiferum*.

Only small amounts of a non-gelatinous material were obtained from the strains of *F. plauti* and the VPI strains 11018, 11936C and 12188 (Table 1). These preparations did not contain the LPS markers 3-hydroxy-tetradecanoic acid and heptose. GLC analysis indicated only small or minute amounts of α -

detected

TABLE 3 Fatty Acids in LPS from *F. necrophorum*, *F. mortiferum*, *F. gonidiaformans*, *F. varium*, *F. naviforme*, *F. russi*, *F. nucleatum*

Strain	Fatty acids (%)	Mol % of			
		14:0	3 OH 14:0	16:0	3 OH 16:0
<i>F. necrophorum</i>					
VPI 6161	10.4	23.4	76.6		
SPH 1	16.3	25.3	61.3	13.4	
N 167	9.4	22.9	77.1		
<i>F. mortiferum</i>					
VPI 0473	26.4	19.1	65.6	15.3	
VPI 4249	13.8	26.4	47.2	26.4	
VPI 5695	14.9	17.7	66.2	16.1	
<i>F. gonidiaformans</i>					
VPI 0482A	10.1	35.3	64.7		
VPI 4381	11.1	31.4	68.6		
VPI 4879	18.6	17.6	82.4		
VPI 11360	13.4	24.0	76.0		
<i>F. varium</i>					
ATCC 8501	11.1	23.5	76.5		
VPI 0499A	16.1	31.1	68.7		
VPI 4234	19.0	22.5	77.5		
Lille 109	9.6	31.8	68.2		
<i>F. naviforme</i>					
VPI 4877	9.1	21.1	78.9		
<i>F. russi</i>					
VPI 0307	14.0	17.4	68.1	14.5	
<i>F. nucleatum</i>					
F5 F7 F10 F14 F18	6.4-15.2	33.4-37.0	38.5-40.7		23.9-26.9
F1 F3 F6 F9 F11	10.6-17.6	22.7-35.6	28.9-43.9	6.1-17.7	17.9-27.5

a) Some preparations contained 16:0 and a few 12:0 in trace amounts (<1% dry weight)

14:0 *n*-tetradecanoic acid 3-OH 14:0 3-hydroxy tetradecanoic acid

16:0 *n*-hexadecanoic acid 3-OH 16:0 3-hydroxy hexadecanoic acid

DISCUSSION

The presence of the typical LPS constituents 3-hydroxy tetradecanoic acid, KDO and heptose characterized the LPS preparations from *F. necrophorum*, *F. mortiferum*, *F. gonidiaformans*, *F. varium*, *F. nucleatum* (see also 1), *F. naviforme* VPI 4877 and *F. russi* VPI 0307. On the other hand, no constituents typical of LPS could be detected in the extracted materials of the three strains of *F. plautii*, *F. naviforme* strains VPI 10646 and VPI 12188 and *F. russi* strains VPI 10646 and VPI 11018. It is of relevance that the fatty acid patterns of whole

cells of the same bacterial strains did not include 3-hydroxy tetradecanoic acid or other known hydroxy fatty acids (12). The results indicate that particular strains of Gram-negative bacteria can be devoid of LPS, or more likely the LPS is absent with respect to chemical composition. This biologically important aspect merits further study using other extraction methods.

The neutral sugars found in the present study, *L-glycero-D-mannoheptose*, *D-glucose*, *D-galactose*, *D-rhamnose* are common constituents of bacterial LPS. *D-glycero-D-mannoheptose* (heptose) study is less widespread and has

F. nucleatum (1) *Proteus mirabilis* (16) *vulgaris* (25) *Yersinia pestis* (3) *Yersinia enterocolitica* (29) *Veillonella* (6) and as part of soluble LPS of some phototrophic bacteria.

32) The sugar is apparently an intermediate in the synthesis of L-glycero-D-mannoheptose and it has been shown that L- and D-glycero-mannoheptose may be part of the same LPS molecule (7-15). Thus the LPS core structure of *Rhodospirillum rubrum* contains three molecules of L-glycero-D-mannoheptose and one molecule of glycerol-D-mannoheptose (Radziejewska & Le personal communication 1979).

Important finding was the occurrence in the LPS of L-glycero-D-mannoheptose (heptose) as a major component. Within *Enterobacteriaceae* this is characteristic of rough mutants versus the LPS of *F. nucleatum* strain Fev 1 has L-glycero-D-mannoheptose as part of both the accharide core structure and the O-specific accharide (7).

Hydroxy tetradecanoic acid is a common constituent of bacterial LPS (21-36). The presence of n-tetradecanoic acid as the only non-hydroxy fatty acid in significant amounts has been reported for isolates from *Pseudomonas diminuta* (37). The radiographic examination of the *F. nucleatum* confirmed the previous findings of 3-hydroxy tetradecanoic acid as a taxonomic marker of this genus (4, 12). 3-Hydroxy hexadecanoic acid has been found in *F. nucleatum*.

The LPS of *F. mortiferum* VPI 0473 and *F. nucleatum* ATCC 8501 had higher contents of 3-hydroxy tetradecanoic acid than reported earlier (4). The reason is possibly that alkaline hydrolysis gives low yields of hydroxy fatty acids (14) used in the former study.

The results of the present study indicate that the structure of *F. nucleatum* is conserved.

Species examined do not seem to differ markedly in their chemical composition.

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IMMUNOCHEMICAL ANALYSIS OF THE TEICHOIC ACID FROM *STAPHYLOCOCCUS SIMULANS*

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stand A Grov A & Oeding P Immunochemical analysis of the teichoic acid from *Staphylococcus simulans* Acta path microbiol scand Sect B 88 121-123 1980

The wall teichoic acid of *Staphylococcus simulans* has been characterized as a glycerol phosphate polymer with glycosidically linked N acetylglucosamine Susceptibility to β -N acetylglucosaminidase and serological similarity to poly C₃ from *Staphylococcus saprophyticus* showed that the amino sugar is in the β -configuration

Key words *Staphylococcus simulans* teichoic acid immunochemistry

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all skin The species is coagulase negative and from the statement that *S. simulans* teichoic acid contains glycerol and galactosamine, no data have been given on this teichoic acid More knowledge of the teichoic acid is desirable particularly because an overlap has been reported of several properties of *S. simulans* with those of human strains of *S. aureus* (4)

MATERIALS AND METHODS

ains The type strain *Staphylococcus simulans* CCN 2705 (CC 2705 RI -

along those described in (4) were supplied by W. E. Coos (Raleigh USA) Bacteria of these 9 strains were used in agar precipitation experiments

Standard Teichoic Acids (Polysaccharides)

Poly C₆ (α N acetylglucosaminyl glycerol teichoic acid) from *S. hyicus* VA 308 and poly C₃ (β -N acetylglucosaminyl glycerol teichoic acid) from *S. saprophyticus* 3519 were produced according to (2, 7)

Isolation of Polysaccharide

The polysaccharide from *S. simulans* CCN 2705 (poly 2705) was extracted from whole bacteria using 70 mM phosphate buffer pH 6.5 at 37 °C Three extractions each lasting 24 h were performed Acid and ethanol precipitation as well as purification on DEAE cellulose (Whatman Ltd England) and Sephadex G 100 (Pharmacia Sweden) columns were performed as described (7)

Qualitative and Quantitative Analyses

Samples (2-5 mg) of polysaccharide were hydrolysed with 0.5 ml 3N HCl for 3 h at 100 °C or 6N HCl for 20 h at 105 °C in sealed tubes flushed with nitrogen Circular paper chromatography was carried out with the following solvent systems

- A. Propanol NH₃ H₂O (6:3:1 v/v)
- B. Ethylacetate pyridine H₂O (40:11:6 v/v)
- C. Butanol HAc H₂O (4:1:1 v/v)

The examination of sugars and sugar alcohols as described above was of the same nature

determined quantitatively as trifluoro acetylated butyl esters (8) with a glass column packed with Taborb (Regis Chemical Co USA)

Hexosamines were determined quantitatively by a modified Elson Morgan method (3) and phosphorus as described in (10)

Enzymatic Treatment

Polysaccharides (0.05 mg/ml) were treated with 0.03 units/ml β -N acetylglucosaminidase (Boehringer BRD) in 100 mM sodium citrate buffer pH 4.4 at 37 °C for 40 h (7). After treatment the reaction mixture was neutralized and then subjected to double diffusion

Serological Methods

Immune sera were raised against formalin killed bacteria by intravenous injections into New Zealand white rabbits (5). Double diffusion in agar was performed as previously described (1). Reaction between polysaccharide and concanavalin A (Con A) (Sigma Chemical Co USA) was carried out as described in (7).

RESULTS

The polysaccharide was eluted from the DEAE cellulose at a molarity of KCl between 0.37 M and 0.42 M. Approximately 100 mg of purified polysaccharide was obtained from 100 g of wet bacteria.

Paper chromatography of 3N HCl hydrolysates of the purified polysaccharide in solvent system A showed the presence of glycerol in addition to glycerol 1 phosphate using periodate benzidine as detecting reagent. No ribitol could be detected. Chromatograms sprayed with molybdate reagent showed only two spots: one corresponding to glycerol 1 phosphate, the other to glycerol diphosphate.

Paper chromatography of 3N HCl hydrolysates in solvent system B and C showed the presence of an amino sugar as detected by the silver nitrate and ninhydrin reagents. The amino sugar was identified as glucosamine by gas liquid chromatography. No galactosamine could be detected. The amounts of phosphorus and N acetylglucosamine estimated by colorimetric analyses were 6.3 per cent and 10.3 per cent respectively; the molar ratio of N acetylglucosamine to phosphorus thus being 0.23 to 1.

Quantitative analysis of amino acids by gas liquid chromatography showed the presence of the usual peptidoglycan amino acids: viz. alanine, glycine, glutamic acid and lysine, the amounts being 0.07, 0.09, 0.08 and 0.04 μ moles/mg respectively.

Antiserum to *S. simulans* CCM 2705 bacteria produced one precipitation line against purified poly 2705. This line showed a reaction of identity with

one of the several lines produced against CCM bacteria.

Antiserum to *S. saprophyticus* 3519 precipitation line against poly 2705 which crossed completely with the line produced by poly C₂ crossed the antiserum/poly 2705 line. No line observed between antiserum 2705 and poly C₁ was any line observed between antiserum *hyicus* VA 308 and poly 2705.

Treatment of poly 2705 with β -N acetylglucosaminidase destroyed its serological activity with antiserum 3519 but not against antiserum. The enzyme destroyed the serological activity of poly C₂ but not that of poly C₁.

Poly 2705 and poly C₂ produced no line with Con A, whereas poly C₁ did.

Antiserum 3519 produced a precipitation line fusing with that of poly C₂ with bacteria CCM 2705 and 7 of the 9 additional *S. saprophyticus* strains tested.

DISCUSSION

The teichoic acid from *S. simulans* CCM 2705 N acetylglucosaminyl glycerol teichoic acid is indicated by the demonstration of glycerol 1 phosphate, glycerol diphosphate and glucosamine. The reported demonstration of galactosamine in the teichoic acid of this strain (4) is not in accord with our results. Glucosamine was the only sugar present in the poly 2705 preparation. Our results showed that the glucosamine is β -glycosidically linked. Both the susceptibility to β -N acetylglucosaminidase and the lack of reaction with Con A support this. Furthermore, the serological reaction with poly C₂ shows that these teichoic acids have common antigenic determinants.

One difference between poly 2705 and poly C₂ is the degree of sugar substitution. The sugar substitution in poly 2705 was found to be only 20 per cent, whereas 50 per cent of the glycerol residues of poly C₂ are sugar substituted (2). The effect of N acetylglucosaminidase on the limited number of sugar determinants in poly 2705 eliminated the serological reaction with serum 3519 but had no apparent effect on the poly 2705 line, which is likely due to the glycerol phosphate.

Serological differences due to different degrees of sugar substitution have been demonstrated (2, 7). The amount of antibodies with specificity against the glycerol phosphate seems to depend on the degree of sugar substitution. The 2705 serum apparently contains antibodies primarily directed against the glycerol phosphate but not the antibody against the sugar.

by C_{β} has earlier (6) been demonstrated antigenically in several strains belonging to different species of *Staphylococcus* recently described (4-9). Presence of the poly C_{β} precipitation line in 11 of the 10 *S. simulans* strains tested is in accordance with this. β -N-acetylglucosamine thus appears to be the immunodominant determinant of teichoic acids of most of these species.

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EPIDEMIOLOGY OF *PSEUDOMONAS AERUGINOSA* INFECTION IN PATIENTS TREATED AT A CYSTIC FIBROSIS CENTRE

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184 isolates of *P. aeruginosa* were obtained from the respiratory tract of 45 out of 70 cystic fibrosis
(CF) patients at monthly examinations during one year at the Danish CF Centre. All isolates were sero-
grouped (O antigens) and phage typed and in this way 99 per cent of the isolates could be grouped
and/or typed. The isolates from CF patients belonged to many different sero groups but 55 per cent
were polyagglutinable and most of these belonged to the 0-3/9 complex. This was significantly
different from the isolates from healthy controls.

The 0-3/9 complex was isolated from 10 (22 per cent) of the patients, belonged
to the 0-3/9 complex and was lysed by phage 109 either alone or in combination with a few other
phages. Furthermore in a few cases the eradication of another strain of *P. aeruginosa* by chemotherapy
was followed by colonization of the lungs with the above mentioned strain.

Key words: *Pseudomonas aeruginosa*; epidemiological markers; sero-grouping; bacteriophage typing;
cystic fibrosis; cross infection.

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Pseudomonas aeruginosa, notably mucoid strains,
is frequently in the respiratory tract of cystic
fibrosis (CF) patients in contrast to most other
types of patients (16). The reason for this
association between CF and *P. aeruginosa* is not
known but the possibility of cross infection exists
to suggest the prevalence of cross infection.

Abstract

Isolates

Isolates were kept separate from each other (3,
28).

The aim of the present study was to investigate
by means of O grouping and phage typing the
epidemiology of *P. aeruginosa* infection in the CF
patients observed at the Danish CF Centre during
one year.

MATERIALS AND METHODS

Patients and Bacterial Material

In this work a *P. aeruginosa* isolate indicates the
descendants of a pure culture of *P. aeruginosa* isolated
from a single specimen from a patient. A *P. aeruginosa*

strain indicates several separate isolates from one or more patients of *P. aeruginosa* assumed by O grouping and phage typing to be descendants of the same isolate.

During the period of this study (January 1st to December 31st 1973) about 90 CF patients were observed at the Danish CF Centre as reported previously (10). These patients are examined as outpatients each month or further during hospitalization and bacteriological examination of sputum or laryngeal secretion is carried out as reported previously (10, 13). During the one year period of the study 551 isolates of *P. aeruginosa* were obtained from 551 sputum or laryngeal secretions from 53 of the CF patients (13) and 484 (88 per cent) of these isolates from 45 patients (85 per cent) were available for the present study.

Age and sex distribution of the CF patients: 21 females, 24 males, mean age 9 years, range $\frac{1}{2}$ –25 years. 36 of the CF patients were chronically infected (≥ 6 months continuously) and 9 intermittently (10).

Twenty seven of the patients are still alive (1978) whereas 18 have succumbed. From 35 of the patients (78 per cent) *P. aeruginosa* was isolated one or more times two years later (1975) and these isolates have also been O grouped and phage typed to see whether the same epidemiological types were still present in the patients. All the *P. aeruginosa* isolates were stored at room temperature in the dark in meat extract agar slants with sealed corks as reported previously (3, 25).

The control material consisted of 501 *P. aeruginosa* isolates from blood cultures from 501 patients with bacteraemia from hospitals all over Denmark submitted for typing during 1969–1977.

Mucoid non mucoid variation is not dealt with in this paper since this phenomenon has been analysed in several papers from this laboratory (3, 11, 13, 15, 16, 18).

Typing and Grouping Procedure

All isolates originating from each patient were typed and grouped on the same day. One subculture was made on 5 per cent blood agar before the typing procedure.

Sero grouping. This was performed by slide agglutination with live antigens from an overnight 5 per cent blood agar culture. The antisera corresponding to Habs O groups 1–12 (19) have been described previously (24, 25). Self agglutinating isolates (SA) agglutinated in saline and non groupable isolates (NG) were not agglutinated by any of the sera. When interpreting the results the main rule was that if monoagglutinable isolates were serologically different then they were considered truly different (1). However this rule cannot be strictly adhered to because of the related 0–2/0–5 antigens and the related 0–7/0–8 antigens (19, 26). Furthermore many of the *P. aeruginosa* isolates from CF patients are polyagglutinable (agglutinated by more than one serum) (1, 21) and it has been shown that lysogenization can change a monoagglutinating into a polyagglutinating strain (sero conversion) (19, 21). In such cases therefore the main method for distinguishing between different strains is not sero grouping but phage typing.

Bacteriophage typing was carried out by *in situ* and typing set of 22 bacteriophages (phages 7, 14, 31, 44, 68, 73, F7, F8, F10, 109, 119r, 35⁺, 1, M6, C2, C11, C18, C21, 188) obtained from the Infection Laboratory, Colindale Avenue, London. The isolates were not lysed by the phage concentration of RTD (routine test dilution) or titrations of 100 \times RTD were used. When the phage patterns were interpreted isolates were allowed by as much as two strong reactions with one being regarded as different (1, 2). Isolates which were not lysed by any of the phages by any of the were designated NTU.

The predominating strain belonged to the complex 3/9 (see Table 2) and was lysed by phage either alone or in combination with other phage patterns; this strain is referred to as 0–3/9 np.

Precipitating Antibodies against *P. aeruginosa*

Sera from all CF patients were examined for the presence of precipitating antibodies against *P. aeruginosa* by means of crossed immunoelectrophoresis as described previously (11, 16). The largest amount of precipitating antibodies determined in each patient during the period was used for the calculation of the titre.

Carbenicillin resistant isolates

Sensitivity to carbenicillin was determined during the period of study by means of Na⁺ (Rosco a/s Tåstrup, Denmark) as described by the manufacturer (direct method). Isolates were considered to be resistant to carbenicillin if the MIC calculated from the regression curve of the manufacturer was ≥ 16 mg/ml. The composition of the sensitivity medium is reported previously (20).

Statistical Methods

The fourfold table test and the Mann-Whitney U test were used. Level of significance: 5 per cent (two-tailed tests) (5).

RESULTS

Sero grouping

About 95 per cent of the 484 isolates were O grouped (Table 1) but more than half of the isolates were polyagglutinable. The most prevalent O group was the 0–3/9 which was harboured by more than half of the patients. In 10 (22 per cent) of the patients there was a shift of sero group between 0–3 or 0–3/9 complex (see legend to Table 2).

Table 2 shows the prevalence of infectious episodes caused by the different O groups of *P. aeruginosa* in CF patients and in outpatients serving as control. Polyagglutinable isolates belonging to the 0–3/9 complex predominated in CF patients in contrast to the control where 0–6 was the most prevalent O group.

TABLE 1. Distribution into O Groups of 484 *P. aeruginosa* Isolates Cultured from 45 Cystic Fibrosis Patients during one Year

Group	Number of Isolates		Number of Patients Harboured the Isolates
0	4	(40%)	4
1	38 (18%)		11 (24%)
2	10		5
3	5		2
4	73 (15%)		10 (22%)
5	4		2
6	2	(55%)	2
7	7		2
8	1		1
9	25		6
10	1		1
11	207 (43%)		25 (56%)
12	1		1
13	1		1
14	5		1
15	3		1
16	10		4
17	11		1
Aggl. (SA)	23	(5%)	8
Non-groupable (NG)	3	(0.5%)	3

* See footnote to Table 2

majority of the isolates from the control group were agglutinable and most of these belonged to the usually related 0-2/0-5 groups and the mutually used 0-7/0-8 groups. The distribution into O groups of a large number of non-bacteraemic *P. aeruginosa* isolates from non-CF patients is similar to that of bacteraemic isolates presented in this paper (Rosendal manuscript in preparation).

Phage Typing

The number and percentage of *P. aeruginosa* isolates lysed by the individual phages used for typing *P. aeruginosa* are shown in Table 3. Fifty per cent of the isolates were lysed by phage 109 and 39.4% by phage 1-43.

Combined O grouping and Phage Typing

When these two procedures were combined only five (1 per cent) of the isolates originating from five CF patients were non-groupable and non-typable. When the results of the remaining 479 isolates were analysed 13 different epidemiological strains of *P. aeruginosa* were present in more than one CF patient (Table 4) thus indicating that cross-infections have probably occurred between these patients. These cross-infecting strains comprise a total of 48 per cent of all the 484 *P. aeruginosa* isolates and the non-

P. aeruginosa infection. This epidemiological *P. aeruginosa* strain was rare in the control material (< 1 per cent).

Nine of the CF patients harboured *P. aeruginosa* intermittently during the one year period of the study and three of the chronically infected patients were temporarily free from *P. aeruginosa* infection.

Of the patients the epidemic 0-3/9 type 109 ± strain

strain indicates several separate isolates from one or more patients of *P. aeruginosa* assumed by O grouping and phage typing to be descendants of the same isolate.

During the period of this study (January 1st to December 31st 1973) about 90 CF patients were observed at the Danish CF Centre as reported previously (10). These patients are examined as outpatients each month or further during hospitalization and bacteriological examination of sputum or laryngeal secretion is carried out as reported previously (10, 13). During the one year period of the study 551 isolates of *P. aeruginosa* were obtained from 551 sputum or laryngeal secretions from 53 of the CF patients (13) and 484 (88 per cent) of these isolates from 45 patients (85 per cent) were available for the present study.

Age and sex distribution of the CF patients: 21 females, 24 males; mean age 9 years, range 1/2–25 years; 36 of the CF patients were chronically infected (≥ 12 months continuously) and 9 intermittently (10).

Twenty seven of the patients are still alive (1978) whereas 18 have succumbed. From 35 of the patients (78 per cent) *P. aeruginosa* was isolated one or more times two years later (1975) and these isolates have also been O grouped and phage typed to see whether the same epidemiological types were still present in the patients. All the *P. aeruginosa* isolates were stored at room temperature in the dark in meat extract agar slants with sealed corks as reported previously (3, 25).

The control material consisted of 501 *P. aeruginosa* isolates from blood cultures from 501 patients with bacteraemia from hospitals all over Denmark, submitted for typing during 1969–1977.

Mucoid non mucoid variation is not dealt with in this paper, since this phenomenon has been analysed in several papers from this laboratory (3, 11, 13, 15, 16, 18).

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Bacteriophage typing was carried out by phage and typing set of 22 bacteriophages (phages 7, 16, 31, 44, 68, 73, F7, F8, F10, 109, 119, 357, 1, 4, M6, C2, C11, C18, C21, 188) obtained from the CF Infection Laboratory, Colindale Avenue, London. If the isolates were not lysed by the phages at a concentration of RTD (routine test dilution) of 100 \times RTD were used. When the phage patterns were interpreted, isolates were allowed to be as much as two strong reactions without necessarily being regarded as different (1, 2). Isolates not lysed by any of the phages by any of the RTD were designated NTU.

The predominating strain belonged to the O complex 3/9 (see Table 2) and was lysed by phage either alone or in combination with other phages in patterns; this strain is referred to as 0–3/9 type.

Precipitating Antibodies against *P. aeruginosa*

Sera from all CF patients were examined for the presence of precipitating antibodies against *P. aeruginosa* by means of crossed immunoelectrophoresis as described previously (11, 16). The largest amount of precipitating antibodies determined in each during the period was used for the calculations study.

Carbenicillin resistant Isolates

Sensitivity to carbenicillin was determined during the period of study by means of Naarm (Rosco a/s Tåstrup, Denmark) microdilution manufacturer (direct method). Isolates were considered to be resistant to carbenicillin if the MIC was ≥ 16 mg/ml. The regression curve of the manufacturer was used. The composition of the sensitivity medium has been reported previously (20).

Statistical Methods

The fourfold table test and the Mann-Whitney U test were used. Level of significance: 5 per cent (2-tailed tests) (5).

RESULTS

Sero grouping

About 95 per cent of the 484 isolates could be grouped (Table 1) but more than half of these were polyagglutinable. The most prevalent O group was the 0–3/9 which was harboured by more than half of the patients. In 10 (22 per cent) of the patients there was a shift of sero group between 0–3 or 0/11 or the 0–3/9 complex (see legend to Table 1).

Table 2 shows the prevalence of sero-infectious episodes caused by the different O groups of *P. aeruginosa* in CF patients and in another group of patients serving as control. Polyagglutinable isolates belonging to the 0–3/9 complex predominated in CF patients, in contrast to the control group where 0–6 was the predominating O group.

Group	Number of Isolates		Number of Patients Harbouring the Isolates
	4		4
	88 (18%)	(40%)	11 (24%)
	10		5
	5		2
	73 (15%)		10 (22%)
	4		2
	2		2
	7		2
	1		1
	25		6
	1		1
	207 (43%)	(55%)	25 (56%)
	1		1
	1		1
	5		1
1/9	3		1
1/9	10		4
3/3/9	11		1
Aggl (SA)	23	(5%)	8
Group (%)	3	(0.5%)	3

* Footnote to Table 2

ority of the isolates from the control group were agglutinable and most of these belonged to the usually related 0-3/0-5 groups and the mutually related 0-7/0-8 groups. The distribution into O groups of a large number of non-bacteraemic *P. aeruginosa* isolates from non-CF patients is similar that of bacteraemic isolates presented in this paper (Rosendal manuscript in preparation).

Phage Typing

The number and percentage of *P. aeruginosa* isolates lysed by the individual phages used for typing *P. aeruginosa* are shown in Table 3. Fifty per cent of the isolates were lysed by phage 109.

100 per cent of the 45 CF patients harboured *P. aeruginosa* strains which could be lysed by phage 109. Each isolate was lysed on an average by four phages (range 1-14) which is not different from the control group and 190 patterns of lysis were found. Only four per cent of the CF isolates were non-lysable.

Combined O grouping and Phage Typing

When these two procedures were combined only five (1 per cent) of the isolates originating from five CF patients were non groupable and non typable. When the results of the remaining 479 isolates were analysed 13 different epidemiological strains of *P. aeruginosa* were present in more than one CF patient (Table 4) thus indicating that cross infections have probably occurred between these patients. These cross infecting strains comprise a total of 48 per cent of all the 484 *P. aeruginosa* isolates and the most prevalent (104 isolates = 21 per cent) was the 0-3/9 type 109 ± strain which was isolated from 10 (22 per cent) of the CF patients with *P. aeruginosa* infection. This epidemiological *P. aeruginosa* strain was rare in the control material (< 1 per cent).

Nine of the CF patients harboured *P. aeruginosa* intermittently during the one year period of the study and three of the chronically infected patients were temporarily free from *P. aeruginosa* in

these patients the epidemic 0-3/9 type 109 ± strain

TABLE 2 *Percentage of Infections Caused by Different O Groups of P aeruginosa Isolated from a Group of Fibrosis Patients Treated in the Danish Cystic Fibrosis Centre and a Group of Non Cystic Fibrosis Patients with Bacteraemia from Hospitals all over Denmark*

Group	Percentage of Infections Caused in	
	CF Patients	non CF Patients with Bacteraemia
0-1	4.4	11.2
0-2 ^{a)}		1.8
0-3	12.1	10.0
0-4	5.5	2.0
0-5 ^{a)}	2.2	7.4
0-6	11.0	23.0
0-7 ^{a)}		1.2
0-8 ^{a)}		0.6
0-9	2.2	5.6
0-10	2.2	9.2
0-11	2.2	8.2
0-12		
0-1/10		0.2
0-2/5 ^{a)}	6.6	8.0
0-3/9 complex ^{b)}	39.6	2.8
0-5/6		0.2
0-6/10		0.4
0-7/8 ^{a)}		3.8
SA + NG	12.1	4.6
Total Number of Infections	91.0 (100%)	501 (100%)

^{a)} Some authors (19-26) do not consider isolates agglutinable by 0-2 + 0-5 and 0-7 + 0-8 as polyagglutinable. The 0-2 and 0-5 antigens as well as the 0-7 and 0-8 are related.

^{b)} The 0-3/9 complex includes the related polyagglutinable strains 0-1/3 0-3/6 0-3/9 0-6/9 0-7/9 0-3/5/6 0-3/6/9 0-2/3/5/9 as strains isolated subsequently from individual patients chronically infected. These strains shifted frequently between some of these serogroups.

replaced the original strain in two cases following successful chemotherapy against the original strain.

On an average two different strains (range 1-4) judged by combined O grouping and phage typing were isolated from each patient during the one year period. However in patients harbouring more than one strain during the year one of the strains predominated and on an average 77 per cent of the isolates (range 50-96 per cent) from each of these patients belonged to the predominating strain.

P. aeruginosa strains were investigated from 35 of the CF patients two years later (1975) and 27 (77 per cent) still harboured the same epidemiological type whereas eight (23 per cent) harboured another type and three of these had contracted the 0-3/9 type 109 ± strain.

Correlation between Antibody Response and group of P aeruginosa

On an average CF patients harboured *aeruginosa* of the 0-3/9 complex had a significantly ($p < 0.05$) longer duration of the *P. aeruginosa* infection (mean 1.8 years range 1/3-6 years) ($p < 0.02$) larger number of precipitins (mean = 16.2 range 0-51) as compared with other O groups (mean 0.8 years range 0-3 years mean number of precipitins 6.4 range 0-21). Furthermore CF patients harboured more than one epidemiological *P. aeruginosa* during the one year period of the study significantly larger ($p < 0.05$) number of precipitins (mean = 16.5 range 0-51) as compared with non CF patients (mean = 1.2 range 0-10).

terriophage	Number of Isolates Lysed
7	68
16	37
21	54
24	13
31	114 (24%)
44	131 (27%)
68	176 (36%)
73	21
F7	100 (21%)
F8	108 (22%)
F10	0
109	268 (55%)
119x	88
352	115 (24%)
1214	151 (31%)
M4	36
M6	9
C2	57
C11	114 (24%)
C18	9
C21	36
188	127 (26%)
on Typable (NTu)	20 (4%)

CF patients harbouring only one strain (mean =
8.6 range 0-29)

Correlation between Lethality of the Patients and Epidemiological Types of *P. aeruginosa*

CF patients harbouring polyagglutinable strains
had a significantly higher lethality (52 per cent) as
compared to patients harbouring only monoagglu-
tinable strains (14 per cent $p = 0.05$). Furthermore
CF patients harbouring *P. aeruginosa* strains of the
0-3/9 type 109± complex had a significantly
higher lethality (59 per cent) compared with patients
harbouring other strains (22 per cent $p = 0.05$).
These results are based on an observation period of
five years.

Correlation between Resistance to Carbenicillin and Epidemiological Types of *P. aeruginosa*

Eighty four (42 per cent) of the 198 isolates
which were tested against carbenicillin were resis-
tant to that drug. However, these carbenicillin
resistant isolates were distributed into many diffe-
rent epidemiological types without any remarkable
difference from the distribution into types of the
remaining carbenicillin sensitive isolates.

DISCUSSION

Although the 12 O grouping sera used in the
present work do not cover all the O groups of *P.*

TABLE 4. O Groups and Phage Types of Epidemic *Pseudomonas aeruginosa* Strains Isolated From Two or More
Cystic Fibrosis Patients

roup	Phage Type	Number of Isolates	Number of Patients Harbouring the Isolates
1/9	109± ¹⁾	104	10
3	68/F7	42	4
2/5	7/44/68/F8/109/119x/1214/M4/C11/C21/188	29	3
1	68	3	3
4	24/44/F8/109/352/1214	2	2
2/5	68/73/119x/C2/C11	6	2
2/5	1214/188u ²⁾	7	2
2/5	7/16/21/44/68/F7/109/1214/M6/C2/C21	10	2
-6	31/C11/188	9	2
-6	7/24/68	7	2
-6	119x	2	2
-6	7/16/21/31/44/68/F8/109/119x/1214/M4/C11/C21/188	5	2
-6	109/M4	4	2

¹⁾ 109± designates strains lysed by phage 109 either alone or (in short patterns) in combinations with other phages
²⁾ typed at 100xRTD

aeruginosa (19), 95 per cent of the 484 isolates in the present work could be O-grouped, and the additional phage typing left only 1 per cent of the isolates as non-groupable and non-typable, thus underlining the value of the combined grouping- and typing-procedure. In this connection, it should be stressed that the predominance of a single epidemiological type in the material from CF patients is not due to lack of discrimination of the methods used, since the control material and previously published results from this laboratory showed a completely different distribution of epidemiological types of *P. aeruginosa* (25).

In contrast to the control group of other patients, nearly half of the isolates from CF patients in the present material were polyagglutinable. This is in accordance with results obtained by other authors dealing with *P. aeruginosa* from CF patients and other chronic respiratory diseases (19, 21, 26, 28). It has been questioned by Pitt & Erdman (26) whether the antigens responsible for these cross agglutinations are O-antigens, although they are heat-stable. However, according to our investigations (12, 14) only the polysaccharides are heat-stable, and they contain the O-antigens, so it would seem likely that the polyagglutination is caused by antigenic determinants on the O-antigen-carrying polysaccharides. The problems of polyagglutinability concerned almost exclusively the predominant 0-3/9 complex.

During continuous infections in the individual patients, subsequent isolates frequently changed polyagglutinability, although the phage typing results were identical. Since polyagglutinability may be caused by lysogenization and can take place both *in vitro* and *in vivo* in experimental animals (19, 21) our results indicate that lysogenization and subsequent seroconversion actually take place *in vivo* in chronically infected CF patients.

Another property of *P. aeruginosa* isolated from these patients is the ability to produce mucoid substance (3, 13, 27) which also seems to be phage-dependent (14, 21, 22). There is positive correlation between these two independent bacterial properties and the antibody response of the host. The complex antibody response to multiple antigens from *P. aeruginosa* is most pronounced in patients harbou-

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strains with these two properties (3, 17). It is not likely that selection by means of chemotherapy has played any role, since a combination of carbenicillin and tobramycin is used to treat these infections, and the occurrence of carbenicillin-resistant strains was not correlated to the epidemiological type in

accordance with other authors (23) and vir strains were sensitive to tobramycin (unpublished data). This is in accordance with published laboratory experiments (21).

From an epidemiological point of view, the present results show a high prevalence of the type 109 strain among the patients at the Centre. This strain is seldom found in hospitals in Denmark, which would indicate that cross-infection takes place between the CF patients in the centre, as pointed out previously (15). Accumulations of endemic strains of *P. aeruginosa* have been reported from other CF centres (6, 9, 27), but the endemic types were different from the 0-3/9 type 109 strain at our centre.

The subsequent isolation of several epidemiological types from the individual CF patients with one predominating strain has also been observed by other authors (4). This is possibly a reflection of the high rate of cross-infection between these patients.

The 0-3/9 type 109 strain is responsible for most of these cross-infections. This strain is especially virulent for our CF patients, associated with prolonged infection and increase of the lethality in our CF patients (18). This is not a special feature of the endemic *P. aeruginosa* strain at our CF centre; other authors (7) have described a similar and prevalent *P. aeruginosa* strain of another CF centre. However, despite the existence of endemic *P. aeruginosa* strains, the prevalence of CF patients treated in such centres is much lower than that of CF patients treated outside the country (8) and CF patients treated outside the country also contract *P. aeruginosa* infection (Høiby unpublished observations). According to these findings, therefore, it is obviously important to identify and eliminate the routes of cross-infection in CF centres and possibly also other places where CF patients are treated in order to diminish the prevalence of *P. aeruginosa* infection and further reduce the lethality of CF patients.

Miss Annette Frommelt and Miss Annelise B. are thanked for skilful technical assistance.

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accordance with other authors (23) and the strains were sensitive to tobramycin (24, unpublished data). This is in accordance with published laboratory experiments (21).

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OCCURRENCE OF OCHRATOXIN- AND CITRININ-PRODUCING FUNGI ON DEVELOPING DANISH BARLEY GRAIN

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Lillehoj E B & Goransson B Occurrence of ochratoxin and citrinin producing fungi on developing Danish barley grain Acta path microbiol scand Sect B 88 133-137 1980

The distribution of *Aspergillus* and *Penicillium* spp isolates that produce ochratoxin A (OA) and citrinin was studied on developing barley from diverse locations in Denmark during the 1977 crop year From 33 test fields 243 isolates of the two genera were obtained with significant numbers of isolates on the grain at the earliest sampling date (1-2 weeks postanthesis) No specific agronomic factor was linked to the distribution of *Aspergillus* and *Penicillium* spp A taxonomic study of 108 representative isolates of the two genera showed that *A flavus* *P chrysogenum* and *P purpurescens* were the predominant species An investigation of the toxin production capability of the identified species showed that seven strains (6.5%) produced OA and a single isolate synthesized citrinin *P purpurescens* was the dominant OA producing species with four toxin positive isolates

Key words: Ochratoxin A citrinin *Aspergillus* *Penicillium* barley

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Ochratoxin A (OA) and citrinin are toxic metabolites of several *Aspergillus* and *Penicillium* (7-9) Although the toxins have been detected in a number of commodities in geographically diverse locations the principal interest has involved diets of cereal feed grains in Scandinavia and food in certain Balkan countries (7-9, 14) OA is a major cause of porcine nephropathy (9) a usually-occurring disease in Denmark (7-8) and Sweden (14) Presence of OA in feed samples from barley has been linked to increased occurrence of the swine disorder (7-9)

A kidney disease of humans in certain areas of Bulgaria Rumania and Yugoslavia has been related to ingestion of OA (9) A study of foods in these regions has identified a high incidence of the toxin (3) In addition an association between the kidney disease and enhanced rate of urinary tract tumors has been reported (11)

The fungi that produce OA and citrinin are widely distributed and have been identified in many foods and feeds (8-9) The microbes that produce the toxins have traditionally been characterized as «storage» or «postharvest» fungi because their ecological niche is determined by water activities routinely observed in mature grain (11) Since recent studies have shown that storage fungi can infect immature maize kernels in the field, the delineation between storage and field fungi is ambiguous (12) A particularly perplexing problem in these investigations of mycotoxin contaminations has been the question of the origin of the initial fungal inoculum Previous investigations of the mycoflora of developing and freshly-harvested barley have demonstrated a limited occurrence of *Aspergillus* and *Penicillium* spp, but the OA- or citrinin production potential of the isolates was not identified (4, 5, 6)

1 Distribution of *Aspergillus* and *Penicillium* spp. Obtained from Danish Barley after Incubation at 10° and 28 °C

Incubation temperature (°C)	Grain Treatment	Number of Fungal Isolates ^a						Totals
		<i>Aspergillus</i>			<i>Penicillium</i>			
		Sample Date			Sample Date			
		1	2	3	1	2	3	
10	A	1	—	—	17	—	5	23
10	K	1	—	2	27	8	21	59
28	A	9	14	20	16	—	9	74
28	K	16	17	16	21	9	8	87
		27	31	38	81	23	43	243

^a Number of *Aspergillus* and *Penicillium* isolates obtained from wash of 100 grains per sample date per field and from 36 grains samples per sample date per field. Two Petri plates of aqueous suspension (A) and 3 of barley kernels (K) were incubated at each temperature of material from each field.

Isolates obtained from all of the fields suggested an average incidence of about 3 isolates per field. In further consideration of aliquot volumes and it is demonstrated that a total of 3750 *Aspergillus* and *Penicillium* isolates were present in the wash of 300 grains from each field. The 146 isolates from all washed kernels (K) indicated an average distribution of 4.4 isolates/field based on the value of the 108 kernels plated per field, which indicated an occurrence of 3.2% of *Aspergillus* and *Penicillium* isolates.

Representative colonies (108) of the 243 *Aspergillus* and *Penicillium* spp. were characterized taxonomically (Table 2). The isolates chosen for identification were selected because of the frequency of occurrence of similar colonies (gross morphology and color). Of the 21 *Aspergillus* spp., 14 were *A. flavus*; none of the isolates produced aflatoxin. The predominant *Penicillium* spp. *P. cyclopium* comprised about one third of the isolates. *P. purpurescens* and *P. chrysogenum* were also common. The three major *Penicillium* spp. provided approximately 60% of all the identified isolates. Tests for production of OA and citrinin by each of the characterized species on washed barley demonstrated that six isolates produced OA at 28 °C and two at 10 °C with only a single citrinin producing isolate (28 °C). The single *A. ochraceus* isolate obtained in the study produced citrinin at both production temperatures; the remaining citrinin-producing species were an unidentified *Penicillium* spp., *P. purpurescens* and *P. verrucosum*. An isolate of *P. granulatum* produced citrinin.

Examination of the origin of the toxin-producing species showed that seven of the eight were obtained from initial incubations of 28 °C with only a single isolate from 10 °C (Table 3). Four of the isolates were obtained from initial aqueous suspensions and four from washed barley grain. An isolate of *P. purpurescens* obtained from 10 °C incubation produced OA only at 28 °C and another isolate of the same species obtained from 28 °C incubation produced OA exclusively at 10 °C. The remaining isolates were all obtained from 28 °C incubations and produced toxin at 28 °C. The seven OA-producing species found on Danish barley represented a 6.5% incidence of the 108 isolates taxonomically identified and a 2.9% occurrence of all of the *Aspergillus* and *Penicillium* spp. obtained in the study. Although no definite association was observed between the origin of toxin-producing species and agronomic practice, toxin producers were obtained from fields with a below average incidence of total *Aspergillus* and *Penicillium* isolates in six of the eight instances.

DISCUSSION

Although the preponderant mycoflora on developing barley grain consisted of "field" fungi (*Alternaria*, *Cladosporium*, *Cephalosporium*, *Fusarium* spp.) a significant incidence of *Aspergillus* and *Penicillium* spp. was present on grain at all stages of development. Of the two preponderant *Penicillium* spp., *P. cyclopium* and *P. purpurescens* only

The current study was undertaken to 1) provide information on the presence of OA- or citrinin producing fungi in developing Danish barley grains 2) elucidate interregional variation in the incidence of the fungi and 3) study agronomic factors that might contribute to selection of toxin producing fungal isolates

MATERIALS AND METHODS^a

Thirty three fields of Danish barley were selected for sampling in 1977 from diverse locations including North Zealand 4 South Zealand 8 Falster 2 Bornholm 2 Funen 2 North Jutland 10 and South Jutland 5 Agronomic information acquired from each test field included barley variety dates of planting and anthesis and pesticide application Samples were collected at three stages of grain maturity 1) 1-2 weeks after anthesis 2) 3-4 weeks after anthesis and 3) physiologically mature seed Three representative locations (10-20 m²) were selected for sampling within each field and 10 heads were collected per location Sample heads from each field were securely wrapped in water repellent bags and transported to Copenhagen for processing In the laboratory grains were carefully removed from heads to avoid inadvertent fungal contamination A 100 grain sample that was representative of the 30 heads from a field was placed in a sterile beaker and immersed in 25 ml of a sterile Triton X (0.01%) solution The suspension was gently shaken for one min with subsequent dilution (1/10) transfer and spreading of a 0.2 ml aliquot on Petri plates containing ME agar (2% malt extract 2% agar pH 4) (3) The diluted suspension provided a microbial population that allowed for effective discrimination of *Aspergillus* and *Penicillium* spp

Test grains were subsequently washed five times with 50 ml of sterile Triton X aliquots from the last wash yielded less than five colonies/plate Six washed barley grains were transferred to each test plate (2% ME) by sterile procedure Four plates of each initial wash suspension and six plates of washed grain were prepared from material obtained from each test field one half of the plates were incubated at 28 °C for 10 days and the remainder at 10 °C for three weeks The 10 °C incubation was employed because Scott *et al* (15) had observed that low temperatures permitted outgrowth of *Penicillium* spp that failed to compete at higher temperatures

isolates obtained from 28 °C incubations were incubated at 28 °C for 10 days and isolates from 10 °C were

subsequently incubated at 10 °C for 3 weeks Isolates obtained from Czapek plates were re-isolated and maintained on Czapek agar slants

Testing individual *Aspergillus* and *Penicillium* OA citrinin and aflatoxin production was accomplished by culturing the fungi on pearled barley Tween 80 water and 50 g barley (300 ml Erlenmeyer flask) for 12 h before autoclave sterilization for 30 min at 121 °C Duplicate flasks of each test were inoculated with 1 ml suspensions of spores and incubated at 28 °C for 10 days and 10 °C for 3 weeks

For thin layer chromatography (TLC) plates coated with 0.5 mm of Adsorbent (TLC plates coated with 0.5 mm of Adsorbent) applied Solvents included 1) benzene acetone and 2) benzene methanol acetic acid (90/10/5 v/v/v) and 3) hexane acetone acetic acid (90/10/5 v/v/v) and 4) ethyl acetate formic acid (60/30/10 v/v/v) Purity of mycotoxins was determined by equivalent test spots with reference standards of pure toxins was verified by formation of the ethyl ester (16)

RESULTS

During the 1977 crop year 243 *Aspergillus* and *Penicillium* isolates were obtained from fields of Danish barley The average number of isolates per field was 7.4 but large variations were observed with numbers isolated from specific fields ranging from 0-22 Barley kernels from specific fields yielded some *Aspergillus* or *Penicillium* isolates The data indicated a definite heterogeneity between fields in numbers of the fungi and no distinct interregional differences The appearance of the fungi was examined by the barley grain at three maturity stages This demonstrated an early presence of the fungi and *Penicillium* spp but no significant differences between sampling periods Although a higher incidence of *Penicillium* spp than *Aspergillus* isolates was observed in material from the sampling period less variation in number of two genera was found at later sampling times

A comparison of the origin of the fungi from either the initial aqueous suspension or washed kernels (k) demonstrated that more isolates were obtained from kernels than from suspensions (Table 1) More *Aspergillus* isolates were obtained from the 28 °C incubation than from the 10 °C incubations but *Penicillium* isolates were often more frequent after incubation at 10 °C 300 grains (100 × 3 sampling times) were examined from each field for the initial wash and kernel

^a Names of companies or commercial products are given solely for the purpose of providing specific information their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned

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TABLE 2 Distribution of *Ochratoxin* and *Citrinin*-Producing Fungal Species Obtained from Danish Barley

<i>Aspergillus</i> and <i>Penicillium</i> spp	Total	Number			
		<i>Ochratoxin</i> Producers		<i>Citrinin</i> Producers	
		10 °C	28 °C	10 °C	28 °C
<i>A. ficum</i>	1	-	-	-	-
<i>A. flavus</i>	14	-	-	-	-
<i>A. fumigatus</i>	4	-	-	-	-
<i>A. ochraceus</i>	1	1	1	-	-
<i>A. repens</i>	1	-	-	-	-
<i>Penicillium</i> spp	3	-	1	-	-
<i>P. charlesii</i>	1	-	-	-	-
<i>P. chrysogenum</i>	15	-	-	-	-
<i>P. corylophilum</i>	2	-	-	-	-
<i>P. cyclopium</i>	28	-	-	-	-
<i>P. funiculosum</i> series	1	-	-	-	-
<i>P. granulatum</i>	2	-	-	-	1
<i>P. nigricans</i> series	1	-	-	-	-
<i>P. purpur- rescens</i>	22	1	3	-	-
<i>P. raistrickii</i>	1	-	-	-	-
<i>P. roquefortii</i>	5	-	-	-	-
<i>P. stoloniferum</i>	4	-	-	-	-
<i>P. thomii</i>	1	-	-	-	-
<i>P. verru- culosum</i>	1	-	1	-	-
Total	108	2	6	-	1

* Number of isolates producing OA or citrinin on sterile barley at 28 °C for 10 days and 10 °C for three weeks

isolates of the latter species produced OA though other studies have shown that both produce the toxin. No *P. viridicatum* strain isolated from developing barley grain. No agronomic factors or interregional differences be linked to the occurrence of OA or producing fungi.

The predominance of *P. purpurrescens* OA producers isolated from the Danish bar somewhat unexpected because earlier in toxin production by the species had been with isolates obtained from meat (2). The in the toxin-production character in the nant species and the total absence of OA in *P. cyclopium* isolates raises a question factors in the microbial milieu that character for toxin production.

The relatively common occurrence of and *P. cyclopium* observed in the current corroborate observations made by Wellin study of Danish barley in 1970. This investigation had also routinely identified *losum* and *P. hordei*, isolates of these species not dominant microbes in the 1977 results suggest that environmental variations between crop years may exert a selective closely related microbes that occur on barley.

Production of citrinin by *P. granulatum* in this study increase the number of fungi that have been reported to synthesize it. However, the paucity of citrinin producers from Danish barley corroborates earlier tions of the limited importance of citr decisive disease determinant in mycoto nephropathy although the metabolite

TABLE 3 Origin of Fungal Isolates that Produced *Ochratoxin A* or *Citrinin*

Fungal Isolate	Isolation ^a Temp (°C)	Production Temp (°C) ^b		Fre	
		OA	Citrinin	Ident No	A and S ₁
<i>P. purpurrescens</i>	10	28	-	1	
<i>P. purpurrescens</i>	28	10	-	11	
<i>A. ochraceus</i>	28	10 28	-	11	
<i>P. purpurrescens</i>	28	28	-	12	
<i>P. purpurrescens</i>	28	28	-	18	
<i>P. purpurrescens</i>	28	28	-	19	
<i>Penicillium</i> sp	28	-	28	21	
<i>P. granulatum</i>	28	28	-	24	
<i>P. verruculosum</i>					

^a 28 °C for 10 days or 10 °C for 3 weeks

EVALUATION OF ELISA IN THE DIAGNOSIS OF *YERSINIA ENTEROCOLITICA* DIARRHOEA IN CHILDREN

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Vesikari T, Granfors K, Maki M & Gronroos P. Evaluation of ELISA in the diagnosis of *Yersinia enterocolitica* diarrhoea in children. Acta path microbiol scand Sect B 88 139-142 1980

In a series of acute gastroenteritis in children including 2 bacteriologically confirmed cases of *Yersinia enterocolitica* infection paired sera from 286 patients were studied for the presence of *Y. enterocolitica* O3 and O9 antibodies using an enzyme linked immunosorbent assay (ELISA). In the 2 cases with positive stool culture of *Y. enterocolitica* O3 a significant rise was seen in each IgM, IgG and IgA class *Y. enterocolitica* O3 antibodies. There were 6 further cases with elevated anti *Yersinia* IgM, these possibly false positive reactions were distinguished from the confirmed cases of *Y. enterocolitica* infection by the absence of IgG and IgA antibodies.

Key words: ELISA, *Yersinia enterocolitica* diarrhoea, gastroenteritis.

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Enzyme linked immunosorbent assay (ELISA)

Yersinia enterocolitica antibodies of different immunoglobulin classes (2, 3) has appeared useful for the retrospective diagnosis of *Yersinia* arthritis. A distinctive antibody pattern with prolonged persistence of *Yersinia*-specific IgG and IgA antibodies is often present in such patients (4). In children the most common form of yersiniosis is an acute abdominal disease with or without arthritis (7). At the acute stage the diagnosis of *Yersinia* may be more readily based on bacteriological but also in these cases the diagnosis may be missed because of inadequate specimen collection or serological techniques. The present study was undertaken to establish whether the newly-developed ELISA which detects IgM, IgG and IgA-class antibody responses would reveal more cases of *Yersinia* infection than bacteriological studies in a series of gastroenteritis in children.

MATERIALS AND METHODS

Sera. The serum specimens for ELISA were derived from a prospective study of gastroenteritis in children aged 0 to 15 years lasting from December 1, 1977 to November 30, 1978. Paired sera were available from 286 patients with acute diarrhoea admitted to the Department of Paediatrics, Tampere Central Hospital. The serum specimens were collected from each upon admission and in convalescent stage 3 to 4 weeks later.

The sera were studied retrospectively under code for *Yersinia enterocolitica* O3 and O9 IgM, IgG and IgA class antibodies using ELISA (see below).

Microbiological studies. From each patient 1 to 3 stool specimens were taken for bacterial and viral studies. At each time of stool specimen collection a microscopic

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to identify enteric pathogens were searched for *Campylobacter*, *Salmonella*, *Shigella*, invasive *E. coli* toxigenic (LT) *E. coli* and pathogenic serotypes of *E. coli*.

Yersinia enterocolitica 0.3 antibodies. In the first case the IgM antibody response was also strong and distinctive, whereas in the second case the IgM antibodies did not increase much. The results were generally similar using the LPS antigen. In addition to the two bacteriologically confirmed cases there were 6 further patients with negative bacteriological studies but with ELISA findings strongly suggesting recent infection with *Y. enterocolitica* (Table 2). In each case there was some rise of anti-*Yersinia* IgM antibodies, measured by whole bacteria antigen and/or the concentration of *Yersinia* IgM in the second serum specimen at least 21%.

With regard to the IgG and IgA-class anti-*Yersinia* antibodies the bacteriologically negative cases were clearly different from the two confirmed cases of yersiniosis. The IgG and IgA antibody concentrations were low and there was little or no rise in them by the convalescent stage (Table 2). When the LPS antigen was used for ELISA the values exceeded 21% in 5 out of 6 cases. No rises were seen in the IgG and IgA class anti-*Yersinia* antibodies using the LPS antigen or *Y. enterocolitica* 0.3 heat-treated antigen. Clinically the two bacteriologically proven cases of yersiniosis had exudative diarrhoea with faecal leukocytes. This is consistent with an enteroinvasive organism such as *Y. enterocolitica* (8). Among the remaining suspected cases there were 2 with fulminant and 4 with nonexudative diarrhoea. Other bacteriological findings in these patients are shown in Table 2.

DISCUSSION

We conclude that serological studies with ELISA cannot reveal any additional cases of yersiniosis in the present series of childhood diarrhoea. Those children with positive bacterial cultures also had a distinctive *Yersinia* antibody response in all immunoglobulin classes. In addition, among the bacteriologically negative patients there were a few cases with no increase in anti-*Yersinia* IgM antibodies but with no concomitant rise in IgG or IgA antibodies. The present findings may provide guidelines for the interpretation of *Yersinia* serology involving different immunoglobulin classes. Until larger materials of acute yersiniosis are studied it seems safe to suggest that for a positive diagnosis of acute *Y. enterocolitica* infection a rise should be detected in each IgM, IgG and IgA anti-*Yersinia* antibodies. A diagnosis of recent *Yersinia* infection based on the demonstration of anti-*Yersinia* IgM antibodies in a single serum specimen must be viewed critically

in the absence of simultaneous high levels of *Yersinia* antibodies of other immunoglobulin classes.

The origin and nature of the 'nonspecific' anti-*Yersinia* IgM antibody increases in the bacteriologically negative cases remains unresolved. The well-known cross reaction between *Y. enterocolitica* and *Brucella abortus* (1) is excluded since this cross-reaction can be avoided by the use of purified LPS antigen (3, 6). It is also unlikely that the observed high titers of *Mycoplasma pneumoniae* CF (Table 2) antibodies in these patients result from true *Mycoplasma* infections. Instead we suggest that both the apparent *Yersinia* IgM- and *Mycoplasma* antibodies represent cross reactions with unknown microbial or other antigens or that they result from general humoral immune stimulation associated with gastrointestinal infection (5).

This study was supported by grants from the Emil Aaltonen Foundation, the Medical Research Council of the Academy of Finland and the Association of Finnish Life Insurance Companies. The technical assistance of Mrs. Soile Nuutsoho, Mrs. Taina Tamminen and Mrs. Tuire Lahti is gratefully acknowledged.

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TABLE 1 *Yersinia enterocolitica* 0 3 IgM- IgG- and IgA class Antibodies Measured by ELISA in Two Clinically Bacteriologically Confirmed *Y. enterocolitica* 0 3 Infection

Patient	Age	Anti <i>Yersinia</i> 0 3 antibody concentration per cent of standard					
		IgM		IgG		IgA	
		I ^{a)}	II ^{b)}	I	II	I	II
1	5 yr 9 mo	7	288	7	>500	4	49
2	3 yr 8 mo	4	21	2	105	6	7

a) I = acute stage

b) II = convalescent stage (4 weeks)

rotavirus and adenovirus. These results have been reported in part previously (9).

ELISA procedure The ELISA method has been described in detail previously (2, 3). In brief, the assay was a double antibody method utilizing immunoglobulin class specific rabbit anti human sera and swine anti rabbit IgG alkaline phosphatase conjugate. The antigen preparations used were formalized whole *Y. enterocolitica* 0 3 bacteria (11), heat treated (100 °C for 1 h) *Y. enterocolitica* 0 9 bacteria, and lipopolysaccharide (LPS) from *Y. enterocolitica* 0 3 (6). All ELISA determinations were made in duplicate. The mean absorbance values of the two determinations were compared to standard sera separate for IgM, IgG and IgA, which had been termed to have a 100% concentration of the corresponding

antibody (2). Accordingly, the results of ELISA expressed as per cent values. The cut-off for a positive reaction for IgM was 21%, and for IgG 7% and 8% respectively. These limiting values obtained by testing negative sera described previously (2).

RESULTS

Y. enterocolitica 0 3 was isolated from 2 of acute diarrhoea in the series. The corresponding anti *Yersinia* 0 3 antibody concentrations measured by ELISA are shown in Table 1. In both cases, there was a very clear increase of both IgG and IgA

TABLE 2 *Yersinia enterocolitica* 0 3 IgM, IgG and IgA class Antibody Concentrations Measured by ELISA in Patients with Acute Diarrhoea, Negative Bacterial Cultures for *Y. enterocolitica* and Possible False Positive Serology

Patient	Age	Anti <i>Yersinia enterocolitica</i> 0 3 antibody concentration per cent of standard						Other microbiologic findings
		IgM		IgG		IgM		
		I ^{a)}	II ^{b)}	I	II	I	II	
3	1 yr 2 mo	39	21	28	9	9	9	rotavirus +
4	3 yr 4 mo	30	39	10	8	19	23	mycoplasma CF I 4096 II 4096
5	3 yr 11 mo	7	22	6	16	4	2	mycoplasma CF I 32 II 256
6	8 yr 2 mo	8	90	3	16	18	25	mycoplasma CF I 4 II 256
7	8 yr 4 mo	13	24	9	10	9	13	invasive <i>E. coli</i>
8	11 yr 4 mo	28	42	13	18	14	21	~

a) I = acute stage

b) II = convalescent stage (4 weeks)

AN ANTIGEN COMMON TO A WIDE RANGE OF BACTERIA

1 The Isolation of a Common Antigen from *Pseudomonas aeruginosa*

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Sompolinsky D Hertz J ■ Høiby N Jensen K Mansá B & Samra Z. An antigen common to a wide range of bacteria. 1 The isolation of a Common Antigen from *Pseudomonas aeruginosa*. Acta path microbiol scand Sect B 88 143-149 1980

In crude water soluble extracts of *Pseudomonas aeruginosa* 64 antigens can be demonstrated by crossed immunoelectrophoresis in agarose with polyvalent *Pseudomonas* immunoglobulin. One of these antigens cross reacts with antigens prepared from bacteria of a wide range of taxonomic groups. Monospecific immunoglobulins to this antigen (Common Antigen) were produced by immunization with the appropriate immunocomplex extracted from agarose. Common Antigen was purified by the combination of two fractionation methods. Precipitation of the crude extract with 18% (w/v) sodium sulfate followed by gel filtration of the supernatant on a Sephadex G 200 column. By this method 35% of Common Antigen from the crude extract was recovered more than half of the fraction electrophoretically pure. Electrophoresis of reduced Common Antigen on a dodecyl sodium sulfate polyacrylamide gel revealed two protein bands with apparent molecular weights of 59-62 000 and 62-65 000 respectively. The untreated antigen however passed a column of Sephadex G 200 with the void volume indicating a substance of high molecular weight ($> 4\ 600\ 000$).

Key words: *Pseudomonas aeruginosa*, Common Antigen, isolation, Na_2SO_4 precipitation, gel filtration, crossed immunoelectrophoresis.

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Many investigators have in recent years shown interest in the occurrence of serological cross reactions between different bacteria. Cross reacting antigens may be of importance as immunogenic agents by inducing protection against infections with remote pathogenic microorganisms (18). Furthermore, if a cross reacting antigen is shared by an entire taxon of higher or lower range, it may be part

of a vital biochemical structure that has been preserved through phylogenesis. A representative of the latter kind of cross reacting antigens is the substance designated OEP of *Pseudomonas aeruginosa* (13). This substance is part of the endotoxin. Another representative is the Enterobacterial Common Antigen, which is a part of the cell wall of *Enterobacteriaceae* (16).

In an investigation of immunological cross reactions between *Pseudomonas aeruginosa* and 36 other bacterial species Høiby reported (7) that 30 of them representing a wide range of gram negative and two gram positive microorganisms showed cross reactions with the antigen represented by

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After removing the polyacrylamide strip the plate was washed, dried, stained and destained as described previously (11)

Determination

Protein content of antigen solutions was determined according to the method of Lowry *et al* (15)

Materials
Sephadex G 200, Sephadex A 25, DEAE and a mixture of proteins of known molecular weight (LMW)

Casein (Indubiose A 31) was purchased from Laboratoire Biologique Française France. Acrylamide was purchased from Bio Rad Laboratories, Richmond, Calif. N,N'-methylenebisacrylamide was purchased from

RESULTS

from agarose seemed in crossed immunoelectrophoresis with St Ag to be monospecific for CA (18)

Purification of CA by Salting out and Sephadex gel filtration

Many of the fractionation techniques applied (ion-exchange chromatography, acid precipitation, ammonium sulfate and sodium phosphate precipitation) fractions with a high concentration of CA were obtained and only 5-15 other antigens were demonstrated by C-18. By combining two or more of these techniques a higher degree of purity could be obtained. It was however remarkable that CA occurred in the same fractions as antigen 37, a saccharide-containing antigen giving a heavy precipitate on C-18 with St Ab in the second dimension gel (9). By gel filtration on Sephadex G 200 CA and antigen 37 were also recovered either in fractions corresponding to the void volume of the column. However, 18% Na₂SO₄ proved very efficient in precipitating antigen 37 while leaving the majority of CA in the supernatant (table 1) together with about 25 other antigens. When this Na₂SO₄ Ag was applied to columns of G 200 CA could be demonstrated by rocket immunoelectrophoresis in a few of the fractions only. The elution volume for peak concentration of CA corresponded to that of Blue Dextran of a mean \log_{10} of 2×10^5 i.e. CA appeared in the void

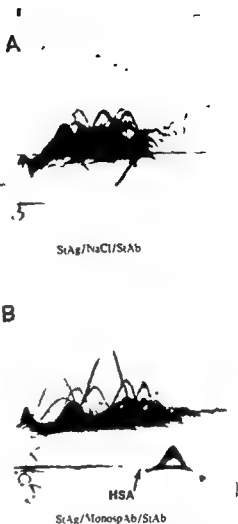


Fig 1 Analysis of the purified and concentrated solution of immunoglobulins G and A to Common Antigen (anti-CA) by crossed immunoelectrophoresis with intermediate gel. The antigen was 2 µl of the polyspecific Standard Antigen throughout.

A Reference system with 20 µl/cm² of the polyspecific Standard Antibody in the second dimension gel and no antigen or antibody in the intermediate gel. Common Antigen is indicated by an arrow.

B As A but with 200 µl anti-CA in the intermediate gel. The mobility of human serum albumin (HSA) is indicated. Staining: Coomassie Brilliant Blue R. Anode to the right (first dimension) and to the top (second dimension).

volumen. In a typical experiment 147.5 mg antigen protein in 10 ml buffer was applied to a 120 x 2.5 cm column of Sephadex G 200. By fused rocket electrophoresis with anti-CA in the intermediate gel CA was demonstrated in 17 fractions of 4 ml

precipitate No 10 of the crossed immunoelectrophoretic reference system for *P. aeruginosa* (8) This antigen will in the present communication be designated Common Antigen (CA) The purpose of this paper is to describe the techniques used to isolate CA from *P. aeruginosa* and the purity and yield of the product achieved

MATERIALS AND METHODS

Reference Antigen and Antibody

The *P. aeruginosa* reference system (St Ag/St Ab) which shows at least 64 different immunoprecipitates in crossed immunoelectrophoresis with intermediate gel (Ciwig) was used as previously described (8, 10)

Preparation of Rabbit Immunoglobulins Specific to CA

After Ciwig with St Ag against an antiserum to *A. baumannii* (7) the precipitate of CA (precipitate No 10 see ref 7) can be recognized even without staining An appropriate part of the agarose containing this precipitate and no other visible precipitates was cut out (4, 20) and washed with isotonic saline for 24 h suspended in dest water and sonicated for 30 seconds (20 000 cps ice cooled) (9) The sonicate was mixed with an equal volume of Freund's incomplete adjuvant and stored at -20°C until use Immunization of three rabbits was performed according to Harbo & Ingild (5) Each rabbit received 100 μl immunogen per immunization and the rabbits were immunized for a period of 12 months A solution of immunoglobulins A and G (anti CA) was prepared as described for St Ab (11)

Antigen for the Isolation of CA

A crude extract of *P. aeruginosa* 888 (O group 5 one of the 4 strains of St Ag) was obtained with a French Press and used for the isolation of CA Thirty grams wet weight of the bacteria suspended in 40 ml dist water was passed once through the press at 10 000 p.s.i. After disintegration the extract was incubated for 30 min at 4°C with deoxyribonuclease and magnesium acetate at final concentrations of 10 $\mu\text{g}/\text{ml}$ and 45 mM respectively (pH 7.4) The remaining whole bacteria and coarse material were separated by centrifugation at $5000 \times g$ for 15 min and cell walls and cell membranes were sedimented at $48000 \times g$ for 30 min The supernatant was passed through a 0.22 μm Millipore® filter

Fractionation of *Pseudomonas* Extract

The crude water soluble extract prepared from *P. aeruginosa* 888 was fractionated by a number of conventional techniques including salting out with various concentrations of sodium phosphate sodium sulfate and ammonium sulfate acid precipitation at pH 4.0 and 4.8 anion exchange chromatography with a chessboard gradient of NaCl and hydrogen ion concentrations and gel filtration on columns of Sephadex G 200 The fractions were analysed for the presence of CA and other antigens by rocket immunoelectrophoresis (21) fused rocket immunoelectrophoresis with interme-

diated gel (1) and Ciwig with St Ab and anti CA result of this analysis the following method was used as a standard procedure for the isolation of CA

a To the crude bacterial extract was added anhydrous Na_2SO_4 per ml The powder soon a compact white flake that was dissolved magnetic stirring (about 2 h at room temp) A heavy turbidity of precipitated material appeared this was separated from the supernatant by filtration The supernatant was dialysed against two of 200 volumes of 40 mM Tris buffer pH 7.2 dialysed solution will in the following be Na_2SO_4 Ag In some cases the sediment of precipitation was dissolved in 40 mM Tris buffer as above and studied separately

b Samples of Na_2SO_4 Ag were applied to a Sephadex G 200 which had been equilibrated with a solution of 0.15 M NaCl 10 mM Tris 0.1% 7.2 The fractions collected were examined by electrophoresis fused rocket electrophoresis intermediate gel and Ciwig The void volume was determined with Blue Dextran (mean MW 2×10^6) & fractions from the G 200 column were further separated on a column of anion exchange gel (Sephadex DEAE) equilibrated with sodium phosphate 1 mM pH 6.0 The antigen was eluted by a constant gradient of NaCl concentration (0-0.8 M) in buffer

Polyacrylamide electrophoresis with Dodecyl Sulfate (SDS PAGE)

SDS PAGE was performed on vertical 8 x 10 cm slabs at 200-250 mA till the bromophenol blue reached the lower edge of the slab The gels were prepared in a buffer of 200 mM Tris 0.1% polyacrylamide and 0.1% N N methylenebisacrylamide were formed in a buffer of 200 mM Tris 0.1% phosphate with 0.2% SDS and 6M urea (pH 8.3) electrophoresis buffer contained 100 mM phosphate 5 mM Na⁺ 5 mM Cl⁻ and 0.1% SDS (pH 8.3) samples were diluted 1:3 and heated to 60°C for 5 min the following buffer 10 mM phosphate 2.5 mM Tris 1.5% dithiothreitol 9M urea pH 7.0 After electrophoresis the slabs were stained with Coomassie Brilliant Blue R 250 in 0.28 M trichloroacetic acid and 10% sulfosalicylic acid during 1 h at 57°C and 57 $^{\circ}\text{C}$ in two portions of trichloroacetic acid (0.65 M) kit of low molecular weight proteins (Pharmacia) used for calibration of the linear best fit relationship between Rf and log of MW (3, 19)

Crossed SDS PAGE Agarose Immunoelectrophoresis

Sample trails of SDS PAGE were washed 20 times at room temperature in the Tris barbiturate buffer used for immunoelectrophoresis Then the slab was placed on a 2.5 cm wide band of 1.5% agarose in 0.05 M Tris barbiturate buffer (pH 8.6) T/2 = 0.5% Triton X 100 on a 7 x 7 cm glass plate The dimension gel contained 1% agarose 1% Triton and 2 $\mu\text{l}/\text{cm}^2$ anti CA The proteins of the SDS PAGE gel were run into the agarose gel

After removing the polyacrylamide strip the plate was washed, dried, stained and destained as previously (11).

Determination

The content of antigen solutions was determined by the method of Lowry *et al.* (15).

As
dextran G 200 Sephadex A25 DEAE and a
of proteins of known molecular weight (LMW
obtained from Pharmacia Fine Chemicals AB
Sweden Urea glycine dodecyl sodium sulfate
sie Brilliant Blue III and Blue dextran 2000 were
ed from Sigma Chemical Co. St. Louis, Miss.
Indubiose A 37) was purchased from
the Biologique Francaise France Acrylamide
Rad Laboratories Richmond, Calif. N,N'-
ne bisacrylamide and sodium sulfate from BDH
als Ltd. Poole, England and Trion X 100 from
Bernsen AS, Copenhagen.

RESULTS

Immunoglobulin to CA

Immunoglobulin to CA (anti-CA) obtained
immunization with the immunocomplex extrac-
from agarose seemed in crossed immunoelec-
oresis with St. Ag. III to be monospecific for CA
(16).

Purification of CA by Salting out and Sephadex gel filtration

Many of the fractionation techniques applied
in-exchange chromatography, acid precipita-
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were obtained and only 5-15 other antigens
demonstrated by C1q-wig. By combining two or
e of these techniques a higher degree of purity
ld be obtained. It was however remarkable that
occurred in the same fractions as antigen 37, a
saccharide-containing antigen giving a heavy
equivalent on C1q-wig with St. Ab. in the second
tension gel (9). By gel filtration on Sephadex
200 CA and antigen 37 were also recovered
either in fractions corresponding to the void
volume of the column. However 18% Na₂SO₄
was very efficient in precipitating antigen 37
while leaving the majority of CA in the supernatant
table 1) together with about 25 other antigens.
When this Na₂SO₄ Ag was applied to columns of
Sephadex G 200 CA could be demonstrated by rocket
immunoelectrophoresis in a few of the fractions.
The elution volume for peak concentration of
CA corresponded to that of Blue Dextran of a mean
m.wt. of 2 x 10⁶. i.e. CA remained in the void

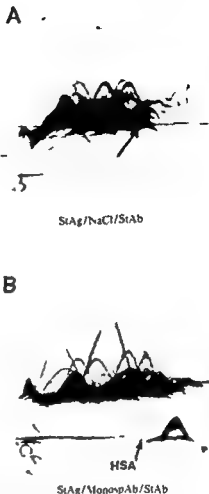


Fig. 1 Analysis of the purified and concentrated solution of immunoglobulins G and A to Common Antigen (anti-CA) by crossed immunoelectrophoresis with intermediate gel. The antigen was 2 µl of the polyspecific Standard Antigen throughout.

A Reference system with 20 µl/cm² of the polyspecific Standard Antibody in the second dimension gel and no antigen or antibody in the intermediate gel. Common Antigen is indicated by an arrow.

B As A but with 200 µl anti-CA in the intermediate gel. The mobility of human serum albumin (HSA) is indicated. Staining: Coomassie Brilliant Blue R. Anode to the right (first dimension) and to the top (second dimension).

volume. In a typical experiment 147.5 mg antigen protein in 10 ml buffer was applied to a 120 x 2.5 cm column of Sephadex G 200. By fused rocket electrophoresis with anti-CA in the intermediate gel CA was demonstrated in 17 fractions of 4 ml

TABLE 1 Isolation of Common Antigen Concentration of Protein and Common Antigen in Various Fractions

	Vol in ml	Protein (mg/ml)	Concentration in % of crude extract	Common Antigen Arbitrary Units per ml	per mg protein	% (from crude)
Crude French Press extract of <i>Pseudomonas aeruginosa</i>	10	14.7	—	100	6.80	100
Na ₂ SO ₄ Ag ¹	10	4.75	32.3	50	10.53	50
Pooled fractions from Sephadex G 200 chromatography ^{2,3}	68	0.055	0.37	5.1	92.7	94
Fraction 24 from Sephadex G 200 chromatography	4	0.126	0.86	13.5	107.14	

¹) Supernatant after precipitation of crude extract with 18% Na₂SO₄

²) The examinations were performed with a mixture of equal volumes of the 17 fractions that contained CA

³) See Fig. 3

each (Fig. 2) Cwlg with individual fractions of antigen in the first dimension and with St Ab in the second dimension gel indicated that 9 of the 17 fractions including those with peak concentration of CA were immunoelectrophoretically pure and in the other 8 fractions only one additional precipitating antigen was demonstrated. When a pool of all 17 fractions was applied to a column of

A25 DEAE and eluted with a gradient of Na⁺ phosphate buffer CA appeared in the fractions 0.3–0.4 M NaCl and no other antigen demonstrated in these fractions.

Apparently pure fractions obtained by G-filtration were pooled and concentrated 5 times an Amicon B15 concentration set (Amicon Co. Lexington Mass.) and 10 µl of the concentrate examined on SDS PAGE. Two bands with close R_F-values were revealed the heavier bands were somewhat dependent on the tube used for denaturation of the sample (Fig. 3). Using 30 min at 60 °C the apparent R_F weights for these substances in different runs

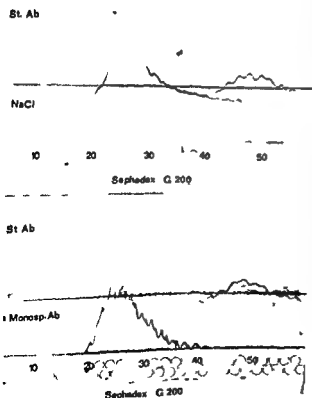


Fig. 2 Fused rocket immunoelectrophoresis with intermediate gel of some of the fractions of Na₂SO₄ Ag¹ from a Sephadex G 200 column (2.5 × 120 cm). The first 130 ml of the eluant fluid was discarded. The collected fractions were 4 ml each.

To each well was applied 5 µl of the fractions and on the figure

A 30 µl/cm² of the polyspecific St Ab in the second dimension gel and no antibody in the intermediate gel.

B 30 µl/cm² of the polyspecific St Ab in the second dimension gel and 30 µl/cm² of the monospecific CA in the intermediate gel. A large precipitate corresponding essentially to the 17 fractions 20–36 has drawn into the intermediate gel and corresponds to Common Antigen. This antigen is the first one from the column. Blue dextran of a mean mol wt of 106 Daltons was eluted in the same fractions as CA Antigen.



polyacrylamide-electrophoresis with dodecyl sulfate. A and D protein markers. B, C, E and

ion with sample buffer at room temperature

30 min
15 min
10 min

ical details see text

ns in E were calculated to 59 700 and 54 000

by Thompson and Kavanagh (1971)

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by salting-out and G-200 filtration. The concentration of CA was estimated by measuring the heights of rockets as compared to varying concentrations of crude extract. Na_2SO_4 -Ag contained 32.3% of the protein of crude extract and 50% of the CA. Whereas the relative total protein concentration was rather constant in different preparations of Na_2SO_4 -Ag, the relative concentration of CA varied between 50–75%. The mean protein concentration of the 17 G-200 fractions which contained CA was 0.055 mg/ml corresponding to 8.8% of the protein applied to the column. The concentration of CA was 5.1% of that of crude extract or 10.2% of that of Na_2SO_4 -Ag. Taking the 6.8 time increase in volume into account, the recovery of CA from the column was about 70% or 35% of the crude extract. Table 1 also shows that in the fraction with peak concentration of CA the relative purity of CA was 15.8 times greater than in the crude extract.

DISCUSSION

Common antigen from *P. aeruginosa* seems to be the bacterial antigen with the widest spectrum of cross reactivity hitherto described. CA corresponds to the High Mobility Antigen from *E. coli* (6) described by Kayser (14) but its biochemical properties differ from those of Enterobacterial Common Antigen (16) or *Pseudomonas* Common Antigen (13). Haub (7) reported cross reactivity of CA with antigens from *Diplococcus pneumoniae*, *Flavobacterium meningosepticum*, *Bacillus cereus*, *Neisseria meningitidis*, *Haemophilus influenzae*, *Bordetella pertussis*, *Pasteurella multocida*, *Escherichia coli*, *Salmonella typhi*, *Shigella sonnei*, *Citrobacter intermedius*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Proteus mirabilis*, *Yersinia enterocolitica*, *Vibrio cholera* and several *Pseudomonas* species. The reference system of *Bordetella pertussis* had a rather similar spectrum of CA (12), whereas the corresponding *N. meningitidis* antigen (no. 19 in ref. 6) in addition cross reacted with strains of *Bacillus subtilis*, *Corynebacterium* sp., *Clostridium perfringens* and *Neisseria catarrhalis*. In neither of the organisms mentioned was cross reaction with *Bacteroides fragilis*, *Staphylococcus aureus* or *Streptococcus pyogenes* observed. Later studies showed no cross reaction with *Mycobacterium phlei*, *M. intracellulare*, an *Actinomyces* sp. or an *Aspergillus* sp. whereas both an *Azotobacter* sp. and *Campylobacter fetus* cross reacted (unpublished results J. B. II and Z. S.). It should be emphasized that in most cases only a single strain from each taxonomic group was

TABLE 1 Isolation of Common Antigen Concentration of Protein and Common Antigen in Various Fractions

	Vol in ml	Protein (mg/ml)	Concentration in % of crude extract	Common Antigen Arbitrary Units per ml	per mg protein	R_f ($R_{f, \text{blue}}$)
Crude French Press extract of <i>Pseudomonas aeruginosa</i>	10	14.7	—	100	6.80	10
Na_2SO_4 -Ag ¹	10	4.75	32.3	50	10.53	5
Pooled fractions from Sephadex G-200 chromatography ^{2,3}	68	0.055	0.37	5.1	92.7	6
Fraction 24 from Sephadex G-200 chromatography	4	0.126	0.86	13.5	107.14	7

1) Supernatant after precipitation of crude extract with 18% Na_2SO_4

2) The examinations were performed with a mixture of equal volumes of the 17 fractions that contained CA

3) See Fig. 3

each (Fig. 2) Cwlg with individual fractions of antigen in the first dimension and with St-Ab in the second dimension gel indicated that 9 of the 17 fractions, including those with peak concentration of CA were immunoelectrophoretically pure and in the other 8 fractions only one additional precipitating antigen was demonstrated. When a pool of all 17 fractions was applied to a column of

A25 DEAE and eluted with a gradient of NaCl phosphate buffer, CA appeared in the fraction 0.3–0.4 M NaCl and no other antigen demonstrated in these fractions.

Apparently pure fractions obtained by G-filtration were pooled and concentrated 5 times on an Amicon B15 concentration set (Amicon Co. Lexington, Mass.) and 10 μl of the concentrate was examined on SDS-PAGE. Two bands with close R_f -values were revealed; the heavier fainter than the lighter one. R_f -values for these bands were somewhat dependent on the temperature used for denaturation of the sample (Fig. 3). Using 30 min at 60 °C the apparent molecular weights for these substances in different runs

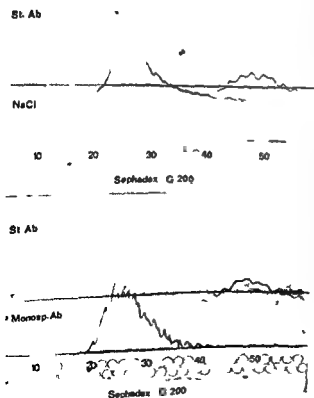


Fig. 2 Fused rocket immunoelectrophoresis with intermediate gel of some of the fractions of Na_2SO_4 Agg from a Sephadex G 200 column (2.5 × 120 cm). The first 130 ml of the eluant fluid was discarded. Collected fractions were 4 ml each.

To each well was applied 5 μl of the fractions indicated on the figure.

A 30 $\mu\text{l}/\text{cm}^2$ of the polyspecific St. Ab in the second dimension gel and no antibody in the intermediate gel.

B 30 $\mu\text{l}/\text{cm}^2$ of the polyspecific St. Ab in the second dimension gel and 30 $\mu\text{l}/\text{cm}^2$ of the monospecific St. Ab in the intermediate gel. A large precipitate corresponding essentially to the 17 fractions 20–36 has been drawn into the intermediate gel and corresponds to the Common Antigen. This antigen is the first one eluted from the column. Blue dextran of a mean mol wt of 106,000 Daltons was eluted in the same fractions as Common Antigen.

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examined. In spite of this the above list of cross-reactions is most impressive.

The present study on the purification of CA was attempted as a part of a project on the biochemical characterization (22) and a study of the physiological function of this substance. By affinity chromatography with antibody coupled to the matrix for the purification of CA a good yield was obtained by elution with SDS-containing buffer (unpublished observations), however, SDS protein complexes are not suitable for some biochemical examinations, and the electrical charge of the eluted protein is altered. It was therefore beneficial that CA proved relatively easy to purify from the crude bacterial extract by a combination of two simple procedures. Precipitation of unrelated antigens with 18% Na_2SO_4 followed by gel filtration on a column of Sephadex G-200. Further purification was obtained by desorption from an anion exchange gel (A25-DEAE). The total yield of purified CA was 35% of the content in the crude bacterial extract. CA proved to be the antigen of the highest mol wt of all antigens not salted out by Na_2SO_4 . CA was eluted with the void volume of the G 200 gel indicating a molecular weight of $> 4-600\,000$. SDS-PAGE showed that it could be dissociated into subunits with molecular weight of 62-65 000 and 59-62 000 Dalton. It is not known whether these bands represent different degrees of degradation of identical subunits or two subunits of different primary structure.

The fractions with the highest concentration of CA from the Sephadex G 200-column (2.5×120 cm) seemed to be immunochemically pure. For example Ciwig of fraction 24 (see Table 1) against the polyspecific St-Ab did not reveal any other precipitate than that of the Common Antigen. The Ciwig was performed with 5 μl antigen in the first dimension gel or 630 ng protein (Table 1). Provided this protein is pure and CA contains only protein then each ml of crude extract contains

$$\frac{100}{13.5} \times 0.126 = 0.93 \text{ mg}$$

Common Antigen per ml or approximately 6%. Anyhow this estimate is provisional and awaits further support by additional techniques.

The technique for isolation of CA by Na_2SO_4 precipitation combined with G-200-chromatography seems satisfactory for preparative purposes. The loss of CA by salting out is of less importance since the procedure is cheap, rapid and simple. The recovery of CA from Sephadex gel filtration is high and its purity in most of the fractions will probably prove sufficient for further biochemical analyses.

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ULTRASTRUCTURAL OBSERVATIONS ON MICROGAMETOGENESIS AND THE STRUCTURE OF THE MICROGAMETE OF *ISOSPORA FELIS*

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Ferguson D J P Birch Andersen A Hutchison W M & Sum J Chr Ultrastructural observations on microgametogenesis and the structure of the microgamete of *Isospora felis* Acta path microbiol scand Sect B 88 151-159 1980

The endogenous forms of *Isospora felis* were observed within the epithelial cells of the small intestine of the cat. They were situated within a parasitophorous vacuole which was limited by a multilaminar wall. The ultrastructural features of microgametogenesis were studied at 8 days post-infection. The initial phase of microgamont development consisted of cytoplasmic growth accompanied by a number of nuclear divisions. The gamont was enclosed by a pellicle and its surface area was greatly increased by deep invaginations. In the later stages of development the numerous nuclei were situated close to the pellicle. Each nucleus has peripherally condensed chromatin. Formation of the microgametes occurred as protrusions from the microgamont surface. Two basal bodies, the dense portion of a nucleus and a mitochondrion entered each protrusion. The microgametes matured while still attached to the gamont from which they finally budded off into the parasitophorous vacuole leaving a large residual cytoplasmic mass. The mature microgamete was found to consist of an elongate nucleus which overlaps with a mitochondrion towards the anterior end of the organism. The anterior portion contains a dense perforatorium and two basal bodies with attached flagella. In addition a number of microtubules (5-9) were found to run longitudinally from the basal body region.

Key words: *Isospora felis* ultrastructure microgametogenesis microgamont cat

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Within the coccidia there have been few reports of the ultrastructure of the endogenous forms of members of the genus *Isospora* (13-22). Recent discoveries have shown that *Toxoplasma gondii*, *Isospora* spp., *Besnoitia* spp. and *Frenkelia* spp. are coccidian parasites which produce *Isospora*-like oocysts (for references see 1-5, 6, 15).

In order to obtain data for a comparison of the ultrastructural features of the endogenous development of the genus *Isospora* with that of the other coccidian parasites we examined the development of *Isospora felis* in the small intestine of the cat.

To the best of our knowledge there have been no reports on the fine structure of microgametogenesis in the genus *Isospora* except for a few observations on the immature microgamont of *I. belli* (22). In the present paper we describe the host/parasite relationship, the process of microgametogenesis and the structure of the microgamete of *I. felis*.

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In two recent reviews on the classification of the coccidia it has been proposed that certain species including *I. felis* should be removed from the genus *Isospora* and placed in a new genus. Two names have been proposed for this genus *Levineia* (1) and *Cystoisospora* (5). Since neither of these names have been generally accepted we will in this report to prevent any confusion refer to the organism studied as *Isospora felis*.

MATERIALS AND METHODS

Specific Pathogen Free cats were infected using sporulated oocysts of *I. felis* administered via a stomach tube. To study microgametogenesis the cats were autopsied at 8 to 9 days post infection. The small intestine was removed and divided into 12 portions. Samples from the portions with the highest parasite density were processed for electron microscopy. The procedure used was similar to that described previously for *Eimeria brunetti* (2). In summary the samples were fixed in glutaraldehyde and osmium tetroxide and embedded in either Vestopal W or Spurr's epon. Thin sections were examined in a Philips EM 200 or EM 300 electron microscope after staining with uranyl acetate and lead citrate. The results are based on the examination of more than 400 micrographs.

RESULTS

Host/Parasite Relationship

The infecting organisms were situated in a parasitophorous vacuole within the epithelial cells of the small intestine. This vacuole lay between the nucleus and the microvilli of the brush border of the host cell. The limiting wall of the vacuole was 30 nm thick and consisted of four electron dense and

three electron lucent bands (Figs 2, 4). It appears that membranes of the host cell reticulum are involved in the formation of (Fig. 2) and its substructure is consistent close apposition of three unit membranes.

As the parasites grew the host cell distended. In addition the host cell nucleus and nucleolus were greatly enlarged (Fig. 13).

During parasite development small amorphous material (approximately 60 nm diameter) were observed within the parasitophorous vacuole adjacent to its limiting wall. The vacuole had formed evaginations into the cytoplasm and a number of the amorphous material were present in these evaginations (Figs 4, 6). Parasitophorous vacuoles containing microgametes possessed fewer and shorter evaginations which contained macrogametes (cf. Figs 1, 11).

Microgametogenesis

Within the epithelial cell the infective meronts underwent dedifferentiation with the loss of anterior organelles and thus formed a trophozoite. Microgametogenesis can be divided into two phases. The initial phase involves cytoplasmic division accompanied by numerous nuclear divisions whereby large multinucleate microgametes were formed. The nuclei were evenly distributed in the cytoplasm and each nucleus had a distinct nucleolus and well dispersed chromatin (Fig. 1). The subsequent phase involved an eccentrically located nucleus with associated centrioles. The cytoplasm of the microgamete contained a number of Golgi bodies, some swollen mitochondria, dense bodies and strands of rough endoplasmic reticulum.

Figs 1-13 are electron micrographs of sections which represent stages in the process of microgametogenesis. They show the ultrastructure of the microgamete of *Isospora felis*.

A double bar (==) on a micrograph represents 1 μ m and a single bar (—) 100 nm.

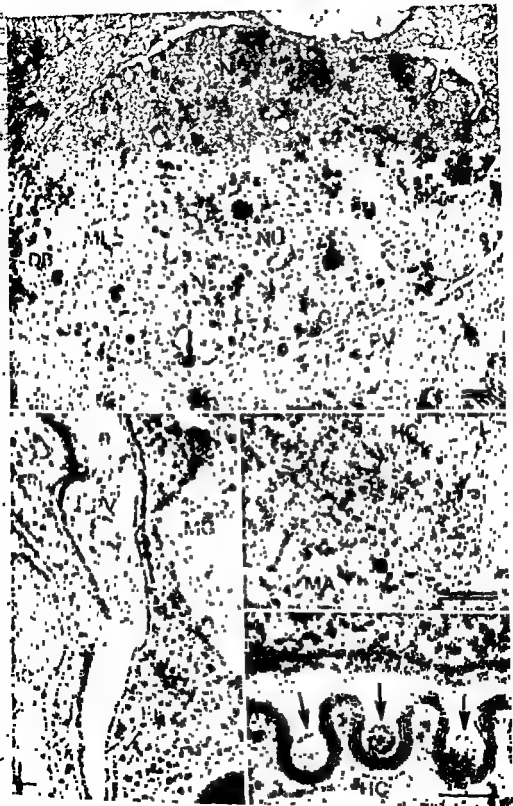
The following abbreviations are used throughout: B = basal body, CE = centriole, DB = dense body, FL = flagellum, G = Golgi body, HC = host cell, HN = host nucleus, MA = macrogamete, MG = microgamete, MI = mitochondrion, N = nucleus, NU = nucleolus, PG = polysaccharide granule, PL = pellicle, PV = parasitophorous vacuole, RM = residual cytoplasmic mass, RN = residual nucleus.

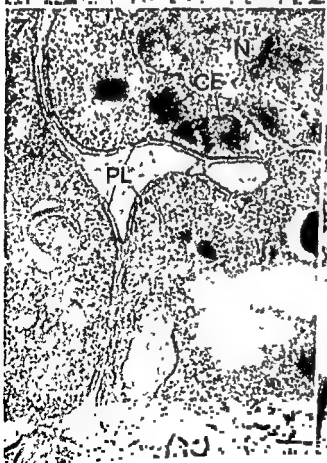
Fig. 1 An early microgamete which possesses a number of evenly dispersed nuclei. The cytoplasm also contains a number of dense bodies, mitochondria and Golgi bodies. Note the number of short invaginations of the parasitophorous vacuole (arrows). $\times 7500$.

Fig. 2 In this section the periphery of a microgamete and part of the host cell are illustrated. The microgamete is limited by a pellicle. Note that the third unit membrane involved in the formation of the multilayered wall of the parasitophorous vacuole is not closely applied to the other two membranes in certain regions (arrows). $\times 9000$.

Fig. 3 A section through part of a host cell containing a microgamete showing the numerous deep invaginations of the wall of the parasitophorous vacuole (arrows) which contain patches of amorphous material. $\times 15000$.

Fig. 4 A high magnification of the wall of a parasitophorous vacuole showing that it consists of four electron dense and three electron lucent bands. Note the evaginations of the wall each of which contains a patch of amorphous material (arrows). $\times 160000$.





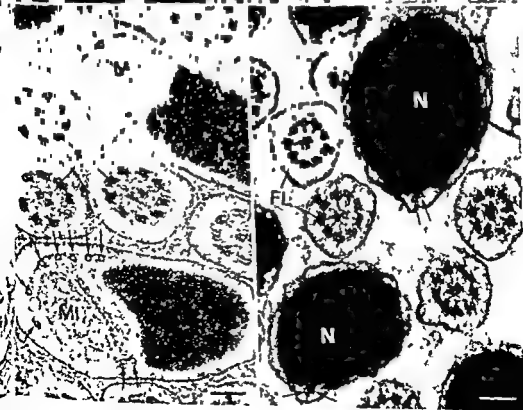


Fig 5 A section through part of a microgamont in which a deep invagination of the pellicle is illustrated. Note that the nuclei are present close to the pellicle or its invaginations $\times 7,500$

Fig 6 This section is through part of a microgamont at a later stage of development than in Fig 5. Note that peripherally located nuclei have the chromatin concentrated into a single dense mass $\times 15,000$

Fig 7 A higher magnification of part of an organism at a similar stage to that in Fig 5 showing that the invaginations are limited by a pellicular structure. Two centrioles situated between a nucleus and the pellicle are also shown $\times 45,000$

Fig 8 A higher magnification of part of a microgamont at a later stage than that in Fig 6. A basal body from which flagellum is protruding into the parasitophorous vacuole is seen. At this stage the chromatin is present as a dense mass at the side of the nucleus adjacent to the basal body $\times 45,000$

Fig 9 A longitudinal section through two developing microgametes which are still attached by their antennae to the microgamont. Note the collar of dense material at each point of attachment (arrows) $\times 45,000$

Fig 10 A section through the anterior part of a microgamete. The presence of a dense plaque which consists of a number of microtubules embedded in a dense matrix is shown (arrows). The two basal bodies with attached flagella are also illustrated $\times 90,000$

Fig 11 A cross-section through two microgametes at the level of the mitochondrial/nuclear overlap. A number of microtubules situated round the periphery of each mitochondrion (arrows) $\times 90,000$

Fig 12 A cross-section through the nuclear region of a number of microgametes. Note the presence of microtubules in each organism at this level $\times 90,000$

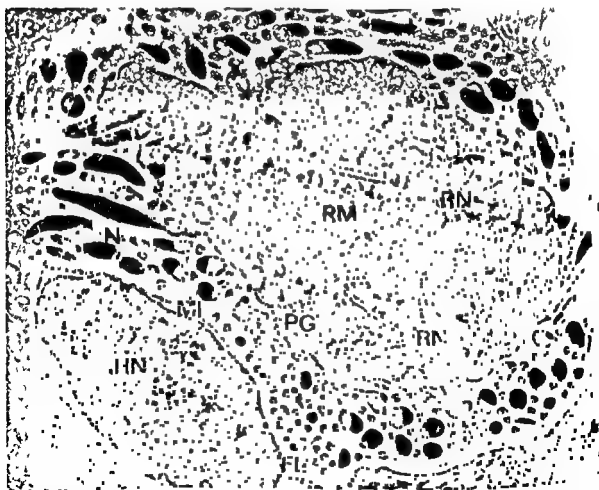


Fig 13 A section through a mature microgamont showing the numerous microgametes within the parasitophorous vacuole and the large residual cytoplasmic mass which contains a number of residual nuclei and polysaccharide granules $\times 7,500$

large number of free ribosomes (Fig 1) about this development the organism remained by a pellicle consisting of an outer unit membrane and an inner layer of two closely applied membranes (Fig 2) At this multinucleate stage the pellicle possessed a few micropores*) and a few invaginations had started to develop (Fig

3) In the later stages the surface area of the organism was greatly increased by deep invaginations of the pellicle (Fig 5) The final nuclear division produced smaller nuclei which lacked a nucleolus and had the chromatin aggregated into a few dense patches at the periphery (Figs 5-6) In addition it was noted that the nuclei were held close to the pellicle (Figs 5-6) Normally microtubules were present between the nuclei and the membranes of the pellicle (Fig 7)

The second phase of microgametogenesis consisted in the actual formation of the microgametes. The microtubules at each nucleus appeared to be directed to basal bodies from which the flagella developed (Fig 8) Each flagellum then grew out into a parasitophorous vacuole (Fig 8) At this stage the inner layer of the pellicle had started to rupture. As this occurred the chromatin was

Each microgamont was very large and could give rise to several hundreds of microgametes

The mature microgamete was crescentic in shape and consisted of a dense elongated nucleus which showed overlapping with a mitochondrion towards the anterior end of the organism (Figs 9-11) The nucleus was enclosed by two closely applied membranes and the mitochondrion possessed several rows of bulbous cristae. The anterior part of the organism consisted of two basal bodies which were embedded in a dense matrix and to each of which a flagellum was attached (Fig 10) The two flagella kept the microgamete close to the anterior end. A dense plaque probably the perforatorium was found close to the anterior tip and in certain sections it was possible to show that it consisted of a number of microtubules embedded in a dense matrix (Fig 10)

A number of microtubules appeared to originate in the basal body region and were observed to run longitudinally through the microgamete. They were distributed around the mitochondrion and their number varied between 5 and 9 in the region of the mitochondrion/nuclear overlap (Fig 11) Normally only two microtubules appeared to extend to the posterior tip of the microgamete (Fig 12)

DISCUSSION

The host/parasite relationship observed in our study of *I. felis* is similar to that reported by Pelster (13). Also the multi-membranous structure of the wall of the parasitophorous vacuole is similar to that described for the endogenous forms of *T. gondii* (14), *I. rivolta* (13) and *S. fusiformis* (18). This is in contrast to the vacuole enclosing the endogenous forms of *Eimeria* spp. which is limited by a single unit membrane (2, 12). The involvement of the endoplasmic reticulum of the host cell in the formation of the parasitophorous vacuole in *I. felis* is similar to that reported for *S. fusiformis* (18) and *S. suhomunus* (11). The functional significance of the patches of dense material present within the evaginations of the parasitophorous vacuole is unknown.

In the microgametogenesis of *I. felis* the initial growth phase with nuclear division and chromatin changes is similar to that described for *Eimeria* spp. (3, 7, 10, 17, 19, 20, 21, 23), *T. gondii* (4, 14), *Sarcocystis* spp. (24, 25) and *I. belli* (22). However in the case of *S. suhomunus* studied *in vitro* nuclear division did not occur prior to the initiation of microgamete formation (11). A retention of the intact pellicle was observed for the developing microgamont of *I. felis*. This is different from what

Microgamete formation now continued by a division of the limiting membrane of the organism.

It remained in the cytoplasmic mass as a

was attached by its anterior end to the microgamont (Fig 9). At this stage a number of microtubules and a dense perforatorium were formed within the developing microgamete. At maturity the microgametes budded off into a parasitophorous vacuole and a large residual cytoplasmic mass which contained a number of saccharide granules was left behind (Fig 13).

*The micropores are not illustrated in this paper but their structure and occurrence is similar to those in previously published observations on microgametogony *Eimeria brunetti* (3).

Fig 5 A section through part of a microgamont in which a deep invagination of the pellicle is illustrated. Note that the nuclei are present close to the pellicle or its invaginations $\times 7,500$

Fig 6 This section is through part of a microgamont at a later stage of development than in Fig 5. Note that peripherally located nuclei have the chromatin concentrated into a single dense mass $\times 15,000$

Fig 7 A higher magnification of part of an organism at a similar stage to that in Fig 5 showing that the invaginations are limited by a pellicular structure. Two centrioles situated between a nucleus and the pellicle are also illustrated $\times 45,000$

Fig 8 A higher magnification of part of a microgamont at a later stage than that in Fig 6. A basal body flagellum is protruding into the parasitophorous vacuole is seen. At this stage the chromatin is present as a dense mass at the side of the nucleus adjacent to the basal body $\times 45,000$

Fig 9 A longitudinal section through two developing microgametes which are still attached to their anterior parent microgamont. Note the collar of dense material at each point of attachment (arrows) $\times 45,000$

Fig 10 A section through the anterior part of a microgamete. The presence of a dense plaque which consists of a number of microtubules embedded in a dense matrix is shown (arrows). The two basal bodies with attached flagella are also illustrated $\times 90,000$

Fig 11 A cross section through two microgametes at the level of the mitochondrial/nuclear overlap. A number of microtubules situated round the periphery of each mitochondrion (arrows) $\times 90,000$

Fig 12 A cross-section through the nuclear region of a number of microgametes. Note the presence of microtubules in each organism at this level $\times 90,000$

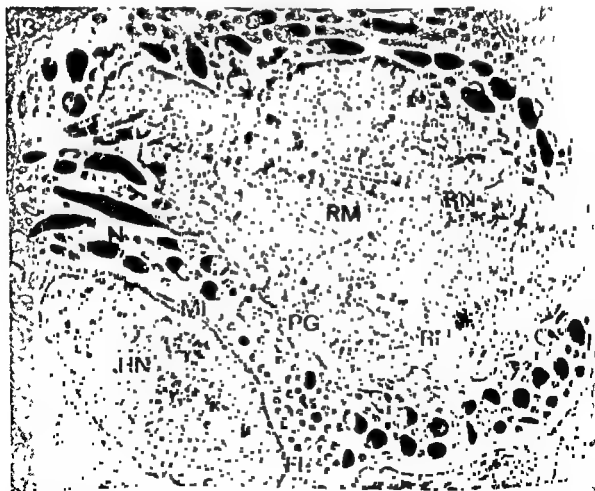


Fig 13 A section through a mature microgamont showing the numerous microgametes within the parasitophorous vacuole and the large residual cytoplasmic mass which contains a number of residual nuclei and polysaccharide granules $\times 15,000$

large number of free ribosomes (Fig 1) throughout this development the organism remained covered by a pellicle consisting of an outer unit membrane and an inner layer of two closely applied membranes (Fig 2). At this multinucleate stage the pellicle possessed a few micropores* and a number of invaginations had started to develop (Fig

3). In the later stages the surface area of the organism was greatly increased by deep invaginations of the pellicle (Fig 5). The final nuclear division produced smaller nuclei which lacked a nucleolus and had the chromatin aggregated into a few dense patches at the periphery (Figs 5, 6). In addition it was noted that the nuclei were situated close to the pellicle (Figs 5, 6). Normally micrioles were present between the nuclei and the membranes of the pellicle (Fig 7).

The second phase of microgametogenesis consisted of the actual formation of the microgametes. The centrioles at each nucleus appeared to be reorganised into basal bodies from which the flagella developed (Fig 8). Each flagellum then grew out through the parasitophorous vacuole (Fig 8). At this time the inner layer of the pellicle had started to invaginate. As this occurred the chromatin was reorganised to be concentrated in a single mass which was situated on the side of the nucleus adjacent to the pellicle (Fig 8).

The formation now continued by a constriction of the limiting membrane of the microgamont in the region directly adjacent to each nucleus. Two basal bodies, the dense portion of a nucleus and a mitochondrion entered each protrusion. As this occurred the dense part of the nucleus was separated from the electron lucent portion which remained in the cytoplasmic mass as a residual nucleus (Fig 9). A collar of dense material was now observed below the limiting membrane of the microgamont at the base of each protrusion (Fig 9). During maturation of the microgamete the dense portion of the nucleus and the mitochondrion became invaginated. This occurred while the microgamete was still attached by its anterior end to the microgamont (Fig 9). At this stage a number of microtubules and a dense perforatorium were observed within the developing microgamete. At maturity the microgametes budded off into the parasitophorous vacuole and a large residual cytoplasmic mass which contained a number of polysaccharide granules was left behind (Fig 13).

*The micropores are not illustrated in this paper but their structure and occurrence is similar to those in previously published observations on microgametogony in *A. bruneti* (3).

Each microgamont was very large and could give rise to several hundreds of microgametes.

The mature microgamete was crescentic in shape and consisted of a dense elongated nucleus which showed overlapping with a mitochondrion towards the anterior end of the organism (Figs 9, 11). The nucleus was enclosed by two closely applied membranes and the mitochondrion possessed several rows of bulbous cristae. The anterior part of the organism consisted of two basal bodies which were embedded in a dense matrix and in each of which a flagellum was attached (Fig 10). The two flagella left the microgamete close to the anterior end. A dense plaque, probably the perforatorium, was found close to the anterior tip and in certain sections it was possible to show that it consisted of a number of microtubules embedded in a dense matrix (Fig 10).

A number of microtubules appeared to originate in the basal body region and were observed to run longitudinally through the microgamete. They were distributed around the mitochondrion and their number varied between 5 and 9 in the region of the mitochondrion/nuclear overlap (Fig 11). Normally only two microtubules appeared to extend to the posterior tip of the microgamete (Fig 12).

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has been reported for *Eimeria* spp where the microgamont is limited by a single unit membrane (3 7, 10, 17, 19 20 21, 23). An increase in surface area produced by invaginations of the limiting membrane has also been reported for the large microgamonts of *E. auburnensis* (7) *E. magna* (10) and *E. brunetti* (3). This feature allows a large number of microgametes to be formed on the microgamont surface. This is in contrast to *I. belli* (22) *T. gondii* (4, 14) *S. suihominis* (11) and *S. singaporensis* (25, 26) which have small microgamonts each giving rise to few microgametes.

The second phase of microgametogenesis in *I. felis* consisting of the formation of the microgametes at the surface of the microgamont occurs in a manner similar to that reported for *Eimeria* spp (3 7, 8 10 17, 19 20 21 23) *T. gondii* (4 14) *Sarcocystis* spp (24 25) and *I. belli* (22). The division of each nucleus into an electron dense portion which enters the developing microgamete and an electron lucid portion which is left behind is also characteristic for *Eimeria* spp (3 7 10 17 19 20 21 23) *T. gondii* (4 14) *Sarcocystis* spp (24 25) and *I. belli* (22). However this does not occur with *E. perforans* (16).

The basic structure of the microgamete of *I. felis* is similar to that recorded for *Eimeria* spp *T. gondii* and *Sarcocystis* spp (3 4 7 8 9 11 14 16 17 19 20 21 23 24 25). A row of microtubules within a dense matrix is present in the anterior region of the organism. This is similar to that reported for *T. gondii* (14) and *E. labbeana* (23) and these microtubules are probably acting as a structural reinforcement. Microtubules running longitudinally from the anterior end of the microgamete have also been reported for a number of *Eimeria* spp *T. gondii* and *Sarcocystis* sp (3 4 9 11 14 17 19 20 21 23 24). The large number (5-9) observed round the periphery of the mitochondrion in *I. felis* is similar to that reported for *E. magna* (21) and *E. maxima* (9). The distribution of these microtubules together with their appearance in reduced number towards the posterior end of the microgamete we feel is consistent with these tubules being concerned with the structural integrity of the microgamete (3) rather than representing the remnants of a third aberrant flagellum (9 19).

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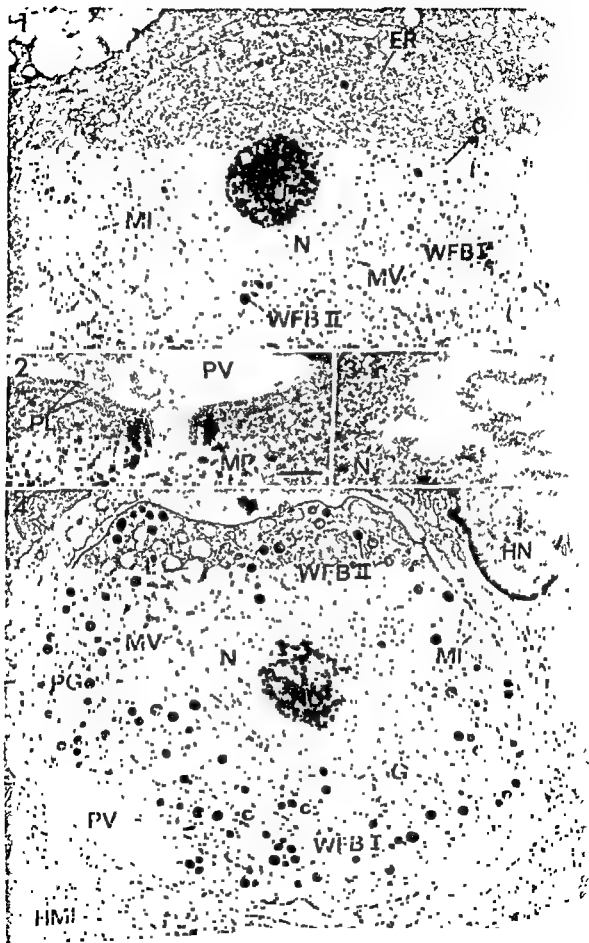
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-12 are electron micrographs which illustrate various stages in the process of macrogametogenesis and the structure of the macrogamete of *Isospora felis*

1 scale bar (=) on a micrograph represents 1 μ m and a single bar (-) 100 nm
 following abbreviations are used throughout C = canalculi ER = rough endoplasmic reticulum \square = Golgi
 MI = host cell mitochondrion HN = host cell nucleus L = lipid globule M = mitochondrion MP = pore
 MV = multimembranous vacuole N = nucleus NU = nucleolus PG = polysaccharide granules PL
 ple PV = parasitophorous vacuole UM = unit membrane V = coated vesicle WFB I = wall forming
 type I WFB II = wall forming body of type II

A longitudinal section through an early macrogamont showing the large nucleus and nucleolus The cytoplasm is a number of peripherally located mitochondria Golgi bodies and amounts of rough endoplasmic reticulum stages in the formation of WFB I and WFB II are also shown $\times 7500$

A high magnification of a section through the periphery of a macrogamont in which the structure of the pellicle adaptation to form a micropore can be seen $\times 90000$

A higher magnification of part of the organism shown in Fig. 4 Note the multimembranous vacuole which to bud off from the nucleus (arrow) $\times 45000$

A section through a macrogamont in which the cytoplasm contains several immature WFB I and II a number mitochondria Golgi bodies polysaccharide granules lipid globules and multimembranous vacuaries The nucleus a large nucleolus is also shown $\times 7500$

A section through part of a macrogamont showing two Golgi bodies with associated membranes of the rough endoplasmic reticulum Coated vesicles are present within the Golgi body on the left while a WFB I can be seen at the of the Golgi on the right An immature WFB II is illustrated within the cisterna of the rough endoplasmic reticulum $\times 30000$

Immature WFB I and II are illustrated in this micrograph The WFB II has an amorphous appearance and is located within a cisterna of the rough endoplasmic reticulum while the WFB I is limited by a unit membrane $\times 9000$

This section illustrates a Golgi body which apparently is involved in formation of WFB I A membrane of the endoplasmic reticulum is present at the base of the Golgi body and a coated vesicle is also observed in this At the apex of the Golgi body a number of large vacuoles are seen which contain material similar to that in WFB I $\times 90000$

A section through two mature WFB I showing that each is limited by a unit membrane In this case the WFB I lay a mottled appearance towards the periphery $\times 90000$

In this section through two mature WFB II it can be seen that they possess an amorphous core with a more cellular cortex Note also that each is situated within a cisterna of the rough endoplasmic reticulum $\times 45000$

A section through part of a late macrogamont in which a deep invagination of the pellicle is present (arrow) $\times 5000$

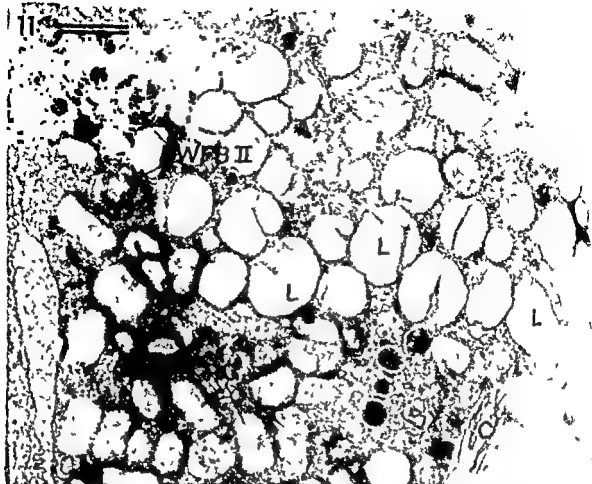
In this section through part of a late macrogamont a group of lipid globules can be seen surrounded by a number of WFB I WFB II and polysaccharide granules $\times 15000$

A section through part of a mature macrogamete At this stage the cytoplasm is filled with numerous polysaccharide granules lipid globules WFB I and WFB II A few mitochondria are also present Note the smooth line of the organism $\times 7500$

In the development of the trophozoite into the macrogamont cytoplasmic growth was not accompanied by nuclear division and the early

be limited by a pellicle which possessed a few micropores (Fig. 2) The cytoplasm of the early macrogamont contained a number of peripherally located mitochondria numerous parallel strands of rough endoplasmic reticulum (rER) many free ribosomes as well as polyribosomes and a number of Golgi bodies (Fig. 1)

At an early stage while the organism was still ellipsoidal in shape the formation of the wall forming bodies of types I and II (WFB I and WFB



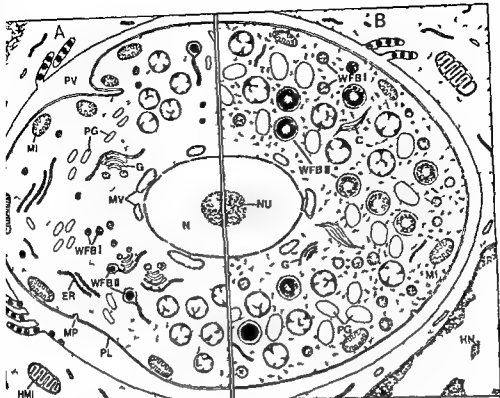


Fig. 1. A diagrammatic representation of the ultrastructure of part of an early macrogamont is given in part A and of a mature macrogamete in part B.

ished micrographs it would appear that the electron lucent wall forming bodies are more likely to represent reserve material (lipid or saccharide) or the coccidia the method of formation of the WFB is incompletely understood. However our observations on *I. felis* are able to provide some morphological information. It appears that the early macrogamont of *I. felis* is well equipped for protein synthesis with large amounts of rER and numerous ribosomes and polyribosomes. Our observations on the formation of the WFB I would be consistent with the passage of protein from the rER to the coated vesicles (transfer vesicles) to the Golgi where it is linked to other substances to produce the material of the WFB I. Such a pathway of synthesis is similar to that previously proposed in *ma* (20) and is compatible with earlier observations in *T. gondii* (6). As pointed out by Heath *et al.* (20) such a process could result in the WFB I containing protein-carbohydrate complexes. This is in concordance with the chemical nature of the outer layer of the oocyst wall which is formed

by the WFB I (13). The WFB II are formed within the cisternae of the rER. This is similar to that reported for *E. mer* spp. (1, 2, 4, 8, 15, 16, 18) and *T. gondii* (6, 12) and probably represents the precipitation of protein molecules formed by the membrane bound ribosomes. The presence of a core and cortex appearance in the mature WFB II is similar to that observed in *I. felis* and *I. rivolta* (11). Although a Golgi body is observed close to the developing WFB II it does not appear to be intimately involved in WFB II formation. This observation differs from that previously reported for *E. bruneti* (4) and *E. acervulina* (7).

We are indebted to Mr J. F. Dunachie and Miss M. Reilly for the maintenance of the SPF cats. We gratefully acknowledge Mrs H. R. R. and Mrs J. Berg for technical assistance and Miss A. G. O. and Mr F. Laursen for photographic assistance. The work was supported by grants from the WHO Geneva, the Wellcome Trust, the Danish Medical Research Council and the W. R. Ross Foundation.

II) was initiated simultaneously in the cytoplasm (Fig. 1). The rER and Golgi bodies seemed to be involved in the formation of the WFB I (Figs. 1-4). Generally a strand of rER was observed close to the base of a Golgi body (Figs. 5-7). The portion of ER adjacent to the Golgi body had lost the ribosomes and a few coated vesicles were present in this region of the Golgi (Figs. 5-7). Vacuoles were now observed at the anterior part of the Golgi. These contained material similar to that within the WFB I and small WFB I were also present close to the Golgi bodies (Figs. 5-7). The WFB I seemed to mature into structures approximately 400 nm in diameter (D) with dense amorphous contents and they were limited by a unit membrane (Fig. 8). In a few cases they displayed a mottled appearance towards the periphery (Fig. 8).

The WFB II were first observed as amorphous spheres 150 nm D within the cisternae of the rER. In many cases a Golgi body was found to be situated close to the rER (Figs. 4-6). The WFB II continued to mature within the cisternae of the rER where the largest could reach a size of 900 nm D (Fig. 9). The mature WFB II situated within distended cisternae of the rER showed a fine amorphous core with a more granular cortex (Fig. 9).

At an early stage of macrogametogenesis changes were observed at the periphery of the nucleus where a number of portions of nuclear membrane and nucleoplasm appeared to be budded off to form multimembranous vacuoles (Fig. 3). These vacuoles contained electron lucent material and remained situated close to the nucleus (Figs. 1-4).

Within the developing macrogamont a store of reserve material in the form of lipid globules and polysaccharide granules was produced (Fig. 4). The lipid globules were the first to mature and could reach approximately 1.2 µm D (Fig. 11). They were normally observed to be situated in small clusters in the cytoplasm of the macrogamont (Fig. 11). The polysaccharide granules first appeared as small rod shaped structures (Figs. 4-11) but as the macrogamont matured they became more spherical in appearance and increased in size reaching 1.2 µm D (Fig. 12).

During the process of macrogametogenesis the number and size of the WFB I and II the polysaccharide granules and lipid globules increased (Fig. 11). During this development the macrogamont presented an irregular outline with a number of invaginations of the pellicle (Fig. 10).

The mature macrogamete was spherical in appearance with a smooth outline and limited by a pellicle. The organism possessed a large nucleus with a nucleolus and the cytoplasm was packed with numerous WFB I, WFB II, polysaccharide granules

and lipid globules (Fig. 12). A number of multimembranous vacuoles were observed close to the nucleus and a few canaliculi, Golgi bodies, mitochondria and a little rER were present within the cytoplasm (Fig. 12).

Text Fig. 1 gives a diagrammatical representation of the ultrastructure of part of an early macrogamont (A) and part of a mature macrogamete (B).

DISCUSSION

The host/parasite relationship of the macrogamete of *I. felis* as observed in the present study is similar to that reported previously (11). It should be noted that the parasitophorous vacuoles lack intravacuolar tubules. This is also the case in *Toxoplasma* sp. (6, 11, 12), *I. rivolta* (11), *Sarcocystis* spp. (19), *Eimeria magna* (16) and *E. fersoni* (11) where intravacuolar tubules are thought to be a characteristic feature of the macrogametes of certain *Eimeria* spp. (see ref. in 10 and 15).

The formation of multimembranous vacuoles from the nuclear surface in *I. felis* has also been reported for *T. gondii* (6) and *E. magna* (16). Vacuoles with a similar appearance have also been observed close to the nucleus in a number of *Eimeria* spp. (4, 7, 8, 16, 18). It is likely that these vacuoles have a common function in the various species but the function is unknown. The vacuoles are also similar in appearance to the dark bodies which have been reported as a new feature of macrogametes of *Sarcocystis fusiformis* (14).

Our observation that the reserve material in a macrogamete of *I. felis* consists of lipid globules in addition to polysaccharide granules differs from that of *Peisteria* (11) where only polysaccharide granules were noted. The development of large amounts of reserve material is also characteristic of the process of macrogametogenesis in *Eimeria* spp. (1, 2, 4, 15, 16, 18) in *Sarcocystis* spp. (14, 21) and in *I. rivolta* (11). These large amounts of food reserves are probably necessary to meet the energy requirements of the process of sporulation.

Within the coccidia the most important characteristic of the macrogamont is the ability to synthesize the wall forming bodies which give rise to the resistant oocyst wall. The macrogametes of *I. felis* are similar to those of *Eimeria* spp. (1, 2, 3, 4, 8, 16, 18), *T. gondii* (6, 12), *I. rivolta* (11), *S. hominis* (9) and *S. fusiformis* (14) in that the types of WFB are present. However, in *Sarcocystis* sp. (19) and *S. singaporensis* (21, 22) only one type of body has been observed. For *I. belli* the situation is unclear. Here two types of WFB have been reported (17): b) a close association of

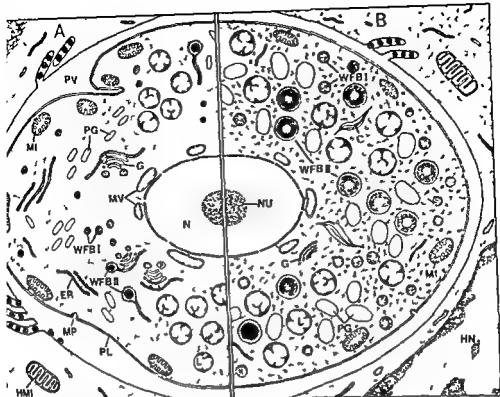


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shed micrographs it would appear that the "electron lucent wall forming bodies" are more likely to represent reserve material (lipid or saccharide).

For the coccidia the method of formation of the WFB is incompletely understood. However, our observations on *I. felis* are able to provide some morphological information. It appears that the early macrogamete of *I. felis* is well equipped for protein synthesis with large amounts of rER and numerous ribosomes and polyribosomes. Our observations on the formation of the WFB I would be

by the WFB I (13). The WFB II are formed within the cisternae of the rER. This is similar to that reported for *Eimeria* spp. (1, 2, 4, 8, 15, 16, 18) and *T. gondii* (6, 12) and probably represents the precipitation of protein molecules formed by the membrane bound ribosomes. The presence of a core and cortex appearance in the mature WFB II is similar to that observed in *I. felis* and *I. rivolta* (11). Although a Golgi body is observed close to the developing WFB II it does not appear to be intimately involved in WFB II formation. This observation differs from that previously reported for *E. brunetti* (4) and *E. acervulina* (7).

Our observations are compatible with earlier observations on *T. gondii* (6). As pointed out by Reilly *et al.* (20) such a process could result in the WFB I containing protein carbohydrate complexes. This is in concordance with the chemical nature of the outer layer of the oocyst wall which is formed

We are indebted to Mr J. F. Dunach and Miss M. Reilly for the maintenance of the SPF cats. We gratefully acknowledge Mrs H. Rain and Mrs J. Berg for technical assistance and Miss A. G. Overgaard and Mr F. Laursen for photographic assistance. The work was supported by grants from the WHO Geneva, the Wellcome Trust, the Danish Medical Research Council and the W. R. Ross Foundation.

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INHIBITION OF HEPATITIS B DANE PARTICLE DNA POLYMERASE ACTIVITY BY PYROPHOSPHATE ANALOGS

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Nordenfelt E, Öberg B, Helgstrand E & Miller E. Inhibition of hepatitis B Dane particle DNA polymerase activity by pyrophosphate analogs. *Acta path microbiol scand Sect B* 88: 169-175, 1980.

DNA polymerase is associated with the core of the so-called Dane particles. The probability that this the hepatitis B viral DNA polymerase offers the possibility of preventing hepatitis B multiplication selective inhibition of this enzyme. We have previously reported that trisodium phosphonoformate (PFA) inhibits Dane particle DNA polymerase. Fifteen compounds with structural similarity to PFA and pyrophosphate have now been tested for inhibition of hepatitis B virus DNA polymerase in an attempt to define the structural requirement for the inhibition. Active structures have two acid groups in close proximity of which at least one is a phosphono group. Phosphonoformate and hypophosphite are the two most active inhibitors. The K_i value for PFA was 7.2 μ M when dTTP was used as substrate and the mechanism of inhibition was non-competitive. Phosphonoformate caused a rapid shut-off of the polymerase reaction indicating that it might inhibit elongation. The efficient inhibition of hepatitis B virus DNA polymerase by PFA and its low toxicity suggest that it could be used to inhibit hepatitis B virus multiplication *in vivo*.

Key words: Hepatitis B DNA polymerase, trisodium phosphonoformate (PFA).

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Evidence available indicated that the so-called Dane particles (6) are the hepatitis B virus. A DNA polymerase is associated with the core of these particles (13-16). The probability that this is the DNA polymerase (18) offers the possibility of preventing hepatitis B virus multiplication by selective inhibition of the Dane particle DNA polymerase.

Intercalating agents like ethidium bromide have been reported to inhibit hepatitis B virus DNA polymerase activity, probably by binding to the DNA, but no selectivity for viral DNA has been shown (11).

In a preliminary communication we have reported that phosphonoformate (PFA) inhibits hepatitis B virus DNA polymerase (21). This finding is here

extended to a determination of the mechanism of inhibition by PFA and an investigation of the structure-activity relation for several PFA analogs. These structures can also be regarded as pyrophosphate analogs.

MATERIALS AND METHODS

Dane Particles

Serum was taken from patients who are chronic hepatitis B antigen (HBsAg) carriers undergoing dialysis. Specimens for DNA polymerase assay were prepared as described earlier (22). In our routine assays 2 ml serum was concentrated to 0.1 ml. In most of the assays in this study the serum source has been one patient (Ass) where 40 ml serum was collected and then concentrated to 0.75 ml and stored in 100 μ l portions at -20 °C.

DNA Polymerase Assay

In the first part of the investigation, assay conditions for the DNA polymerase were as described by Kaplan *et al.* (16) with minor modifications (22). To 25 μ l test sample 2.5 μ l 10% Nonidet P-40, 2.5 μ l 3% mercaptoethanol were added and the mixture kept at room temperature for 30 minutes. This mixture was then added to 100 μ l of a solution of 160 mM Tris pH 7.5, 40 mM $MgCl_2$, 120 mM NH_4Cl , 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP and 20 pmole (methyl- 3H) dTTP (40 Ci/mmole, New England Nuclear Corporation Frankfurt/M) (Method 1). The mixture was incubated at 37 $^{\circ}C$, and at indicated times 20 μ l samples were spotted on CF/C Whatman glassfiber papers. The filters were left in 5% TCA/0.1 M tetrasodium pyrophosphate solution overnight, washed, dried and the radioactivity counted.

During most of the investigation this method was modified, as suggested by Hirschman *et al* (12). The 120 mM NH_4Cl was replaced by 400 mM KCl . Mercaptoethanol was excluded and instead 20 mM dithiothreitol was included in the 100 μl solution added to the sample. Finally, as labelled nucleotide 2.5 μl (^{32}P) dTTP (300 Ci/mmol, 1.0 mCi/ml Radiochemical Centre Amersham, England) was used instead of (^3H)-dTTP (Method 2). These changes gave 10–20 times higher incorporation in cpm compared to the earlier method in tests run in parallel.

The inhibitors were added to the reaction mixture at 4 °C before incubation at 37 °C was started. In all assays duplicate aliquots were taken and the results given in cpm are the mean of observed cpm.

Pyrophosphate Analogs

Tetrasodium pyrophosphate (I) oxalic acid (III) and glycolic acid (VII) were from E. Merck Darmstadt West Germany. Disodium sulphonacetate (XV) was from Eastman Kodak Co. Rochester N.Y. Phosphoglycolic acid (XVII) was from Sigma Chem. Co. St. Louis, Miss. Disodium ethane-1-hydroxy-1,1-diphosphonate (XI) was a gift from The Procter & Gamble Company Cincinnati, Ohio. Disodium hypophosphate (II) was prepared according to Genge *et al.* (9). Trisodium

(XVI) according to Nelson (14) and Johnson & Dunn (2)

Phenylphosphonic acid (XIV) was synthesized according to *Kreuzkamp & Cordes* (17). The method to prepare methyl disodium oxycarbonylphosphonate (V) and sodium ethoxycarbonylphosphonate (VI) will be

published separately (manuscript in preparation) (V) and (VI) contained less than 0.5% PF4. TLC (polyethyleneimine 2M LiCl mobile phase). The structures are shown in Table 2 in their acid form but are ionized at pH 7.5 in the assay.

Determination of Inhibition Constants

Determination of inhibition constants and pK_i of inhibition was according to Cleland (4, 5). A computer programme for plotting regression lines was used.

RESULTS

Time Course of the DNA Polymerase Reaction

Figure 1 shows the time course of a 32 P-dTMP incorporation reaction where 32 P-dTMP incorporation was studied during a 5-hour period. The incorporation was almost linear for 4 hours, both at a concentration of 0.043 μ M dTTP as shown in Fig. 1 and a concentration of 0.986 μ M dTTP which was the highest concentration used in the mechanism.

Inhibition of the DNA Polymerase Activity of Different Patients by PFA

The polymerase activities of Dane particles in sera of 5 patients were compared with respect to inhibition by PFA. As is evident from Table I, PFA inhibited the polymerase activity of all tested sera, e.g. 100 μ M of PFA gave an inhibition between 77-90% and 10 μ M PFA caused inhibition of 39-60%.

Mechanism of Inhibition by PFA

Mechanism of Inhibition by PFA
The rate of inhibition was investigated in experiments where PFA was added to the reaction mixture 30 minutes after the start of the reaction.

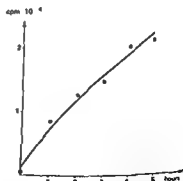


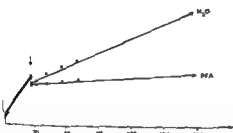
Fig 1 Time-course of the DNA polymerase reaction. 60 μ l of Dane particle preparation, 10 μ l Nonidet P-40 and 400 μ l of a reaction mixture (according to method of [1]) was added. At the designated times after incubation, 20 μ l aliquots of the reaction mixture were removed and analysed by radioactivity counting.

1. Inhibition of DNA Polymerase Activity by PFA Dane Particle Preparations from 5 Patients

Incorporation in the absence of PFA ^b	100 μ M PFA Percentage inhibition	10 μ M PFA Percentage inhibition
7087	89	88
14894	90	61
1150	77	42
584	79	47
4738	80	39

5 μ l serum preparation was added 15 μ l PFA or
and 2.5 μ l 10% Nonidet P 40 immediately before
100 μ l solution (according to method 2)
after 3 hours incubation at 37 °C

shown in Fig. 2 PFA caused an immediate shut
down of the polymerase activity. Fig. 3A shows the
replot of the double reciprocal plot for inhibition of hepatitis B
virus DNA polymerase activity by PFA at varying
concentrations of dTTP. The result indicates that
the mechanism of inhibition is non-competitive. As
shown in Fig. 3B a replot of the intercepts and
slopes gave the inhibition constants $K_i = 7.2 \mu$ M
and $K_{i1} = 22.6 \mu$ M. The K_m value
for dTTP was 0.15μ M as obtained from the
Lineweaver Burk plot.



2. Rate of inhibition of the DNA polymerase
activity by PFA. To 60 μ l of Dane particle preparation
100 μ l Nonidet P 40 and 400 μ l reaction mixture (accord-
ing to method 2) were added.

2. Aliquots were taken immediately before and
after the addition of PFA or H₂O. The reaction rate is
similar both before and after addition. Due to the dilution
30 min a lower rate of reaction is observed after that
time.

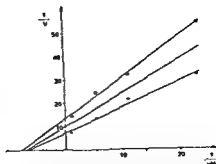


Fig. 3A Double reciprocal plots of the hepatitis B virus
DNA polymerase-catalyzed reaction with dTTP as
variable substrate and PFA as inhibitor. The reaction
was according to method 2 and the incorporation of
(³P)-dTTP during 4 hours was determined. Phospho-
rylphosphate concentrations were 0 μ M (+), 10 μ M (O)
and 20 μ M (●).

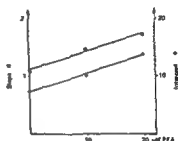


Fig. 3B Replots of slopes (●) and intercepts (+) as a
function of PFA concentration. Data were taken from
Fig. 3A.

Inhibition of Hepatitis B Virus DNA Polymerase by Analogues of PFA

The inhibition of hepatitis B virus DNA poly-
merase activity by PFA and several analogues of
PFA and pyrophosphate is shown in Table 2. A 50%
inhibition was observed at 20 μ M PFA. Hypo-
phosphate (II) inhibited the polymerase activity by
more than 90% at 500 μ M concentration. A slight
inhibition was also seen for pyrophosphate (I) and
carboxydiphosphonate (IX).

The rest of the compounds at up to 500 μ M
concentration did not show any significant inhibi-
tion for the DNA polymerase activity.

DISCUSSION

The hepatitis B virus DNA in the Dane particles
seems to be circular (28) with about two thirds as
double- and about one third as single-stranded DNA.

DNA Polymerase Assay

In the first part of the investigation assay conditions for the DNA polymerase were as described by Kaplan *et al* (16) with minor modifications (22). To 25 μ l test sample 2.5 μ l 10% Nonidet P 40, 2.5 μ l 3% mercaptoethanol were added and the mixture kept at room temperature for 30 minutes. This mixture was then added to 100 μ l of a solution of 160 mM Tris pH 7.5, 40 mM $MgCl_2$, 120 mM NH_4Cl , 0.5 mM dATP, 0.5 mM dCTP, 0.5 mM dGTP and 20 pmole (methyl 3H)-dTTP (40 Ci/mmol, New England Nuclear Corporation, Frankfurt/M) (Method 1). The mixture was incubated at 37 $^{\circ}C$ and at indicated times 20 μ l samples were spotted on CF/C Whatman glassfiber papers. The filters were left in 5% TCA/0.1 M tetrasodium pyrophosphate solution overnight, washed, dried and the radioactivity counted.

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according to Schwarzenbach & Zühlke (10). Tetra sodium ethylenediamine 2M LiCl molybdate spray) gave a single spot with $R_f = 0.10$ and less than 0.5% of PFA. 2-Phenylphosphonoacetic acid (XIV) was synthesized according to Kreuzkamp & Cordes (17). The method to prepare methyl disodium oxycarbonylphosphonate (V) and disodium ethoxycarbonylphosphonate (VI) will be

published separately (manuscript in preparation). (V) and (VI) contained less than 0.5% PFA. 2M LiCl molybdate spray (polyethyleneimine 2M LiCl molybdate spray). The structures are shown in Table 2 in their acid form but are ionized at pH 7.5 as the assay.

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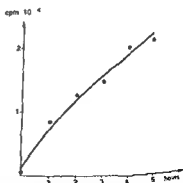


Fig. 1 Time course of the DNA polymerase reaction. 60 μ l of Dane particle preparation, 10 μ l Nonidet and 400 μ l of a reaction mixture (according to method 2) were added. At the designated times after incubation 20 μ l aliquots of the reaction mixture were removed for radioactivity counting.

Compound	Percentage inhibition at 500 μ M
$\begin{array}{c} \text{O} \\ \\ \text{HO}-\text{P}-\text{CH}_2-\text{CH}_2-\text{C}-\text{OH} \\ \quad \quad \quad \\ \text{OH} \quad \quad \quad \text{OH} \end{array}$	14
$\begin{array}{c} \text{O} \\ \\ \text{HO}-\text{P}-\text{O}-\text{CH}_2-\text{C}-\text{OH} \\ \quad \quad \quad \\ \text{OH} \quad \quad \quad \text{OH} \end{array}$	-22

5 μ l serum = 100% inhibition before and after 100% inhibition. All and VII were tested using method I and the others according to method II. Tests were taken immediately and after 3 hours at 37°C.

PFA and a investigation of the mechanism of inhibition by PFA

The hepatitis B virus DNA polymerase reaction was rapidly blocked by PFA (Fig. 2) which might indicate that PFA is an elongation inhibitor. The mechanism of inhibition by PFA was non competitive (Fig. 3A) with regard to dTTP. This is in accordance with the mechanism of inhibition by PFA of herpesvirus DNA polymerase (27), influenza virus RNA polymerase (33) and reverse transcriptase (34). PFA had an inhibitory effect on

the reverse transcriptase from avian myeloblastosis virus with a K_i of 16 μ M when the deoxyribonucleoside triphosphates were varied.

The inhibitory effect of PFA on Dane particle associated DNA polymerase seems to be very specific. There was a 50% inhibition of this polymerase activity at 20 μ M PFA. Only at 100 times that concentration 2000 μ M a 50% inhibition of cellular DNA synthesis has been seen (32). Cellular DNA polymerase γ which also has a low K_m value for dTTP (31) is not inhibited by 500 μ M PFA (B. Eriksson personal communication, 27). No inhibition of bacterial or mitochondrial

The core associated DNA polymerase (13, 16) been reported to initiate DNA synthesis at the end of this partly single stranded DNA (19). Study of this enzyme and its reaction has so far been hampered by the low enzyme activity as measured by incorporation of (3 H)-labelled nucleoside monophosphates and the fact that the Dane particles can only be obtained from patient or chimpanzee sera.

Few attempts have earlier been made to find inhibitors of the hepatitis B virus DNA polymerase. Intercalating agents such as ethidium bromide and quinacrine can inhibit the polymerase activity (11). However, no specificity in binding to hepatitis B virus DNA as compared to cellular DNA has been shown for these compounds. Vidarabine and phosphonoacetate have earlier been reported to be without effect on the hepatitis B virus DNA polymerase (14, 20). In a preliminary communication

the polymerase assay the ease in incorporated radioactivity permitted a more active study of substances similar to

DNA polymerase from several other tested DNA polymerases (10, 27) which are inhibited to the same extent by PFA and PAA. Reverse transcriptase is the only DNA polymerase yet investigated that shows the same pattern of inhibition (34) as Dane particle DNA polymerase. However, in contrast to Dane particle DNA polymerase this enzyme is not inhibited by PFA.

Two compounds hypophosphate (II) and PFA (IV) were effective inhibitors of hepatitis B virus DNA polymerase. Both could be regarded as

Two compounds hypophosphate (II) and PFA (IV) were effective inhibitors of hepatitis B virus DNA polymerase. Both could be regarded as

blocking any of the two acidic groups of PFA led to inactive structures (V, VI). This is in accordance with their lack of effect on herpesvirus DNA polymerase (Eriksson *et al.* manuscript in preparation). Phosphonoacetate (XII, PAA) which has the same effect on herpesvirus DNA polymerase as PFA (10) did not inhibit hepatitis B virus DNA polymerase despite the structural similarity. Of other tested structures at

TABLE 2 *Inhibition of Hepatitis B Virus DNA Polymerase Activity by Pyrophosphate Analogs The Compounds Shown in Their Acid Forms*

Compound	Percentage inhibition at 500 μ M	Compound
I $\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{HO}-\text{P}-\text{O}-\text{P}-\text{OH} \\ \quad \\ \text{OH} \quad \text{OH} \end{array}$	69	IX $\begin{array}{c} \text{O} \quad \text{O} \quad \text{O} \\ \parallel \quad \parallel \quad \parallel \\ \text{HO}-\text{P}-\text{C}-\text{P}-\text{OH} \\ \quad \\ \text{OH} \quad \text{OH} \end{array}$
II $\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{HO}-\text{P}-\text{P}-\text{OH} \\ \quad \\ \text{OH} \quad \text{OH} \end{array}$	97	X $\begin{array}{c} \text{O} \quad \text{OH} \quad \text{O} \\ \parallel \quad \quad \parallel \\ \text{HO}-\text{P}-\text{CH}-\text{P}-\text{OH} \\ \quad \\ \text{OH} \quad \text{OH} \end{array}$
III $\begin{array}{c} \text{O} \quad \text{O} \\ \diagdown \quad \diagup \\ \text{HO}-\text{C}-\text{C}-\text{OH} \end{array}$	28	XI $\begin{array}{c} \text{O} \quad \text{OH} \quad \text{O} \\ \parallel \quad \quad \parallel \\ \text{HO}-\text{P}-\text{C}-\text{P}-\text{OH} \\ \quad \\ \text{OH} \quad \text{CH}_3 \quad \text{OH} \end{array}$
IV $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{P}-\text{C} \\ \quad \diagup \\ \text{OH} \quad \text{OH} \end{array}$	95	XII $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{P}-\text{CH}_2-\text{C} \\ \quad \diagup \\ \text{OH} \quad \text{OH} \end{array}$
PFA $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{P}-\text{C} \\ \quad \diagup \\ \text{OH} \quad \text{OH} \end{array}$		PAA $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{P}-\text{CH}_2-\text{C} \\ \quad \diagup \\ \text{OH} \quad \text{OH} \end{array}$
V $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{P}-\text{C} \\ \quad \diagup \\ \text{OCH}_3 \quad \text{OH} \end{array}$	3	XIII $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{P}-\text{CH}-\text{C} \\ \quad \quad \diagup \\ \text{OH} \quad \text{CH}_3 \quad \text{OH} \end{array}$
VI $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{P}-\text{C} \\ \quad \diagup \\ \text{OH} \quad \text{OC}_2\text{H}_5 \end{array}$	18	XIV $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{P}-\text{CH}-\text{C} \\ \quad \quad \diagup \\ \text{OH} \quad \text{C}_6\text{H}_5 \quad \text{OH} \end{array}$
VII $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{CH}_2-\text{C} \\ \diagup \\ \text{OH} \end{array}$	-10	XV $\begin{array}{c} \text{O} \\ \parallel \\ \text{HO}-\text{S}-\text{CH}_2-\text{C} \\ \parallel \quad \diagup \\ \text{O} \quad \text{OH} \end{array}$
VIII $\begin{array}{c} \text{O} \quad \text{O} \\ \parallel \quad \parallel \\ \text{HO}-\text{P}-\text{CH}_2-\text{P}-\text{OH} \\ \quad \\ \text{OH} \quad \text{OH} \end{array}$	-28	

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500 μ M, only carbonyldiphosphonate (IX) showed a significant inhibition of hepatitis B virus DNA polymerase. These results indicate that active inhibitors have two acid groups at close proximity and that at least one should be a phosphono group. It seems likely that PFA interferes with a pyrophosphate binding site on the polymerase.

The high incidence of hepatitis B virus infections makes the development of efficient chemotherapy important. Interferon (for review see 7), virazole (15) and vidarabine (3, 25) have been tested in clinical trials. The significant but transient effect so far reported for interferon and vidarabine might be improved by longer treatment. However, the toxicity of vidarabine (29) is likely to pose restrictions on the length of treatment with this compound and interferon is still available only in limited quantities. The viral DNA polymerase activity is not inhibited by vidarabine triphosphate (Nordenfelt unpublished observation).

Since PFA has a low cellular (32) and animal toxicity (8) it could be considered as a candidate for clinical trials on hepatitis B. PFA inhibits herpes simplex DNA polymerase *in vitro* and has a therapeutic activity in animals infected with herpes virus (1). It is possible that the Dane particle DNA polymerase activity is necessary for hepatitis B virus multiplication and studies on chimpanzees have been initiated to test this assumption (R. Purcell & K. Tsiquaye personal communications).

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PATHOGENESIS OF SENDAI VIRUS INFECTION IN MICE

On the Possible Role of Interferon on the Development of Disease

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Degre M & Rollag H Pathogenesis of Sendai virus infection in mice On the possible role of interferon in the development of disease Acta path microbiol scand Sect B 88 177-181 1980

Intraperitoneally (i.p.) injected interferon prolonged the survival time of mice inoculated intranasally (i.n.) with Sendai virus and reduced the mortality in mice inoculated i.n. with *Haemophilus influenzae*. Moderate concentrations of interferon were demonstrated in homogenized lungs of Sendai virus infected mice as long as the virus was present. Similar concentrations could be produced by i.p. injection of Sendai virus or interferon. Alveolar macrophages from mice treated i.p. with interferon or Sendai virus phagocytized more actively than control macrophages. From the present and earlier data it is concluded that interferon may have a direct effect on the Sendai virus infection. The total effect of virus pneumonia is a reduction of the lung macrophage antimicrobial activity and therefore the phagocytosis modifying effect of interferon produced in the lungs is probably of minor importance for the outcome of the disease.

Key words Interferon phagocytosis Sendai virus alveolar macrophages

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Parainfluenza 1 virus Sendai virus is a common respiratory tract pathogen in the mouse. Since the naturally transmitted virus usually produces a mild self-limiting infection it is considered an excellent model of human parainfluenza virus respiratory infection.

In addition to the primary virus infection the disease is frequently complicated by secondary bacterial infections complications which may be decisive for the outcome.

The factors with specific and non specific Interferon has long been recognised as a non-specific defence factor against viral infections. Evidence for its role in the total resistance has been presented by many authors although such evidence is mainly circumstantially cases disputable (3).

The present communication is an attempt to analyse the role of interferon in the recovery from murine Sendai virus infection especially the role of the interferon effect on the alveolar macrophage activity.

MATERIALS AND METHODS

Mice Inbred young HaM/ICR/CSF/Bom female albino mice were used for all experiments except those requiring

them

Virus Parainfluenza 1 virus Sendai strain was originally obtained from Dr L. A. Glasgow (University of Rochester Rochester N.Y.). Our strain had been passaged 5 times in embryonated eggs in our laboratory. Virus titres were assayed in embryonated eggs and by the haemagglutination (HA) test using guinea pig erythrocytes.

tes Finally the mouse lethal dose (LD₅₀) was determined by intranasal inoculation into adult mice of 0.1 ml of 10-fold dilutions of virus, 5 mice per dilution. The inoculated mice were observed for 15 days and the mortality was recorded daily. The virus pool, kept at -70 °C, showed an HA titre of 1024 and 5×10^6 egg infectious doses (EID) per 0.1 ml.

Vesicular stomatitis virus (VSV), Indiana strain employed in the interferon test, was propagated and assayed in L-929 cells by the infectivity end point test.

Newcastle disease virus (NDV) was used for induction of interferon. Our strain originally obtained from Dr L. A. Glasgow (University of Rochester, Rochester, N.Y.), was propagated in embryonated eggs and titrated by the HA method, using guinea pig erythrocytes.

Bacteria. For the mortality studies *Haemophilus influenzae* strain b 51, originally obtained from Dr T. Omund (Dept. of Microbiology, Lillehammer, Norway) was grown and used as described earlier (8).

In vitro phagocytosis was performed with *Escherichia coli* strain x-7, sero-group 086, B7 (21).

Interferon. Murine type 1 interferon was produced in L-929 cells, inoculated with NDV. After incubation overnight at 37 °C the supernatant was removed and clarified by low speed centrifugation. The remaining NDV was inactivated by treatment at pH 2 at 4 °C for 4 days. Some of the non-interferon proteins were precipitated by this treatment and removed by centrifugation at 1500 g for 20 minutes. The supernatant was then neutralized to pH 7.2 and used without further concentration or purification. Samples of this preparation were kept at -70 °C and fresh ampoules were used for each experiment. Interferon activity was assayed by the infectivity inhibition microtest (6) employing L-929 cells and VSV as challenge virus. The titres were adjusted to the mouse reference preparation G-002-904-511 obtained from the National Institute of Allergy and Infectious Diseases, Bethesda, Md. The interferon preparation employed in the present study contained 1×10^5 units per ml.

Phagocytosis experiments. Alveolar macrophages were obtained via the trachea (7). The pulmonary lavage was performed with 1.5 and 1 ml of Eagle's minimal essential medium (MEM) containing lignocaine (16), 20 per cent fetal bovine serum and antibiotics. The cells were distributed into Leighton tubes containing «flying coverslips». After incubation overnight at 37 °C in humid atmosphere with 5 per cent CO₂ the non-adherent cells were removed by vigorous washing. The macrophages were re-incubated with the bacterial suspension (10⁷ bacteria per ml in Krebs-Ringer-Glucose buffer without serum as described in detail elsewhere (23)). The tubes were incubated stationarily at 37 °C. After 90 minutes of incubation the «flying coverslips» were removed, washed with physiological saline, fixed with alcohol and stained with Giemsa. The proportion of macrophages containing bacteria and the average number of bacteria per cell were determined microscopically independently by two persons.

Statistical analysis. LD₅₀ was calculated by the method described by Reid & Muench. Comparison of the

mortality rates was made by means of the χ^2 test, employing the 2 \times 2 table analysis.

EXPERIMENTS AND RESULTS

In the first experiment we examined the effect of interferon on the course of the Sendai virus infection. Mice were inoculated intranasally with 0.1 ml of Sendai virus. A randomly-selected half of the group of mice ($n = 32$) were injected intraperitoneally (i.p.) with 1×10^4 units of interferon on 0.1 ml. The injections were started at the same time as virus inoculation and were repeated daily. The other half of the group ($n = 32$) were given the same volume of MEM i.p. daily. The groups were observed for 15 days and the mortality was recorded. The data are summarized in Fig. 1. Final mortality was practically the same in interferon-treated as in control mice (68 and 64.5 per cent respectively). However, mice treated with interferon survived longer (mean 9.8 days) than control (mean 8.3 days). Significantly more control mice died during the first 9 days of observation (17 versus 7) and more interferon-treated mice after day 9 (17 versus 4, $\chi^2 = 8.9$, $p < 0.01$).

The concentration of interferon in the lungs following the i.p. injection of 1×10^4 units was determined. Three mice were killed at designated times after injection. Their lungs were removed aseptically and homogenized in 1 ml MEM. Homogenized lungs were centrifuged at 1500

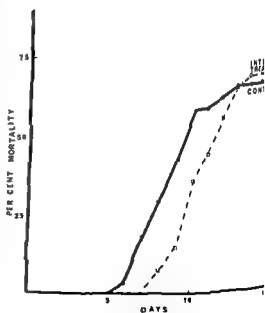


Fig. 1. Mortality of mice following inoculation with Sendai virus. Influence of daily i.p. injection of 1×10^4 units of interferon.

1 Interferon Concentrations in Lung Tissue of Mice Following Intraperitoneal Injection of 1×10^4 Units of Interferon

Hours after injection	Interferon units per ml
0	170 ^a
1	360
2	85
3	10
4	10

^a Mean of three lungs

minutes at room temperature to remove cells and the interferon titres were determined in supernatants. The titres (Table 1) were adjusted to a dilution factor 1.5 (w/v) of the whole lung in MEM. High concentrations of interferon were present in the lungs during the first hour after inoculation and low titres could be demonstrated for the following few hours.

The next experiment was designed to correlate interferon production during the Sendai virus infection to the development of the disease. A group of mice were inoculated with 1 LD₅₀ of Sendai virus. Three mice were killed daily. The lungs were

and high titres persisted to days 7 and 8 and declined rapidly after day 8. The interferon titres correlated well with the virus titres. Both virus and interferon titres declined below detectable levels after day 11.

In the following experiment we measured the effect of interferon on the phagocytic activity of alveolar macrophages. Mice were injected i.p. with 5×10^4 units of interferon or with 0.1 ml of

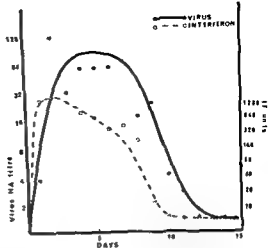


Fig. 2. Virus (HA) titres and interferon (IF) units in the lungs of mice following i.p. inoculation with Sendai virus. Each value represents the mean of three lungs.

Sendai virus containing 1024 HA units per ml. Eight hours later the mice were killed, the macrophages were harvested through pulmonary lavage, then seeded into Leighton tubes and their phagocytic activity was determined. The lungs of mice inoculated with Sendai virus contained $2-5 \times 10^2$ units of interferon at the time when the cells were obtained. The results shown in Table 2 indicate that macrophages from mice injected with either interferon or Sendai virus phagocytized more actively than macrophages from control mice.

To assay the possible role of the enhanced phagocytosis *in vivo*, mice were inoculated i.p. with suspensions of *H. influenzae* in concentrations that produced fatal disease in at least 50 per cent of the mice. One group of mice ($n = 39$) were treated daily with 2.5×10^4 units of interferon i.p. The control mice ($n = 42$) received daily injections of an

TABLE 2. Influence of Intraperitoneal Injection of Interferon or Sendai Virus on the Phagocytic Activity of the Alveolar Macrophages

treatment	Per cent macrophages containing bacteria	Mean number of bacteria per macrophage
control	54.7 (54.0-55.1) ^a	0.95 (0.87-1.38)
interferon 5×10^4 U	75.0 (73.6-76.4)	2.0 (1.8-2.16)
Sendai virus 100 HA U	76.5 (76.1-87.7)	1.66 (1.59-3.65)

^a Median (range) of six counts.

Interferon or Sendai virus was injected i.p. Alveolar macrophages were harvested 8 hours later and their phagocytic activity was determined *in vitro*.

tes. Finally the mouse lethal dose (LD₅₀) was determined by intranasal inoculation into adult mice of 0.1 ml of 10-fold dilutions of virus, 5 mice per dilution. The inoculated mice were observed for 15 days and the mortality was recorded daily. The virus pool, kept at -70 °C, showed an HA titre of 1024 and 5×10^6 egg infectious doses (EID) per 0.1 ml.

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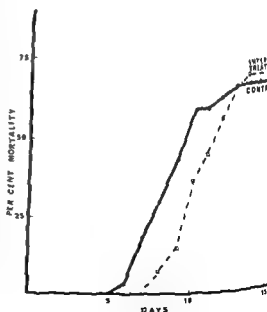


Fig. 1. Mortality of mice following inoculation with Sendai virus. Influence of daily i.p. injection of 1×10^4 units of interferon.

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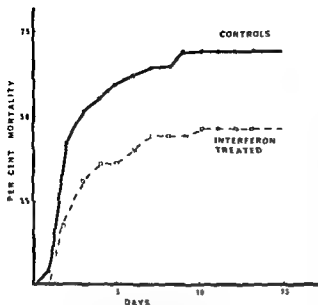


Fig. 3. Mortality of mice following inoculation with *Haemophilus influenzae*. Influence of daily i.p. injection of 2.5×10^4 units of interferon.

identical volume (0.25 ml) of MEM. As shown in Fig. 3 the mortality of interferon treated mice was significantly reduced from 68 to 46 per cent ($\chi^2 = 4.65$, $p < 0.05$). However the treatment did not prolong the survival time of those mice that succumbed to the infection. The mean numbers of days of survival were 3.9 and 3.6 respectively.

DISCUSSION

Being a good model for human respiratory infections the pathogenesis of murine Sendai virus infection has been investigated by several authors. There are a number of host defence factors, both specific and non-specific ones, which probably play some part in the termination of the infection. The decisive role of the specific B cell mediated humoral immune response has been demonstrated (4, 5, 15, 22). Also the T cell and NK cell mediated local response seems to be of importance (1, 2) although it has been questioned whether they are essential for the recovery (15).

Among the non-specific defence factors the bactericidal activities of pulmonary macrophages and the physical transport mechanism of the ciliary epithelium of the bronchial system are both deeply involved in the defence against secondary bacterial infections (8, 12, 14, 18, 19, 20) which are to a large extent responsible for the late mortality following Sendai virus infection of mice (9, 10). Interferon is believed to be a defence factor in primary virus infection also in the respiratory tract (15, 22). In our experiments parenterally injected

interferon prolonged the survival time of Sendai virus infected mice. However these mice are not known to have had interferon in their lungs being induced by the virus. Thus the significance of the additional injected interferon is uncertain.

During recent years it has become evident that interferon exerts a regulatory effect on a wide range of cellular functions (25) including several defence factors against infectious diseases. Moderate concentrations of interferon enhance the phagocytic activity of macrophages *in vivo* and *in vitro* (11, 17, 24) while high concentrations can depress the uptake of bacteria into murine peritoneal macrophages (24).

Interferon injected parenterally in this study reduced rather than enhanced the mortality due to the inoculated *H. influenzae*. The interferon concentration in the lungs following parenteral injection was moderate, comparable to that shown to enhance phagocytic activity (17, 24). It is possible that the phagocytosis-enhancing activity can explain the interferon effect on the development of disease, but other factors may be involved.

The effect of interferon treatment on the Sendai virus infection is more difficult to explain. During the early phase of the infection, i.e. the first 8 days, while virus was present in the lungs, mortality was reduced by interferon treatment, but the late mortality was the same.

It has been repeatedly demonstrated that the microbicidal capacity in the lungs and the phagocytic activity of macrophages are reduced during infection (8, 18, 19, 20, 26). This reduction is usually maximal 4-7 days after inoculation of virus. The mechanism of this effect is not clear. We have attempted to find out whether the interferon production during infection is involved.

During the Sendai virus infection interferon is present in the lungs in the early phase as the virus was detectable in the lungs. The concentrations of interferon are difficult to establish and it is possible that the local concentration in the alveoles, i.e. in the close environment of macrophages, was higher than shown by our results. In *in vitro* studies 5×10^4 units per ml are required to depress significantly the phagocytic activity (24) and it is improbable that concentrations of this magnitude were present in the alveoles. We therefore think that the demonstrated effect of interferon on the phagocytic activity of the alveolar macrophages is of minor, if any, importance for the increased susceptibility of virus infected animals to secondary bacterial infection. The reduction in the phagocytic activity of macrophages can probably be explained by other mechanism(s), e.g. by a direct effect of the virus on the cells.

BRIEF REPORT

TRISODIUM PHOSPHONOFORMATE INHIBITS WOODCHUCK HEPATITIS VIRUS ASSOCIATED DNA POLYMERASE

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Nordenfelt E & Werner B Trisodium phosphonoformate inhibits woodchuck hepatitis virus associated DNA polymerase Acta path microbiol scand Sect B 89 183-184 1980

Recently a new virus has been described which infects woodchucks *Marmota monax*. This virus named woodchuck hepatitis virus (WHV) is closely related to human hepatitis virus (HBV). The viruses have the same principal antigenic system involving surface and core determinants and a serological relationship has been found. WHV has also a DNA polymerase associated with the core. It has previously been reported that trisodium phosphonoformate (PFA) but not phosphonoacetic acid (PAA) inhibits DNA polymerase associated with HBV. This investigation shows the same type of inhibition pattern by PFA and PAA on WHV DNA polymerase.

Key words: Hepatitis B DNA polymerase inhibitor phosphonoformic acid (PFA) woodchuck hepatitis virus.

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Recently a new virus has been described which infects woodchucks *Marmota monax* (Summers *et al* 1978). This virus named woodchuck hepatitis virus (WHV) is closely related to human hepatitis B virus (HBV). Infection with either virus results in the accumulation in the blood of excess viral coat protein in the form of 20-3 nm spherical and tubular particles (Bayer *et al* 1968; Summers *et al* 1978) and 40-50 nm diameter particles (Dane *et al* 1970; Summers *et al* 1978) containing DNA (Robinson *et al* 1974; Summers *et al* 1978) and a DNA polymerase (Kaplan *et al* 1973; Summers *et al* 1978).

A serological relationship has been found between viral antigens of HBV and WHV (Werner *et al* 1979). The viruses have the same principal antigenic systems involving surface and core determinants. The core antigens share major determinants but possess unique antigenic specificities as well. The surface antigens of the

for studying various aspects of the pathogenesis of hepatitis B infections.

It has previously been reported that trisodium phosphonoformate (PFA) but not phosphonoacetic acid (PAA) inhibits DNA polymerase associated with HBV (Nordenfelt *et al* 1979). Further studies on the mechanism of this inhibition have shown it to be of a non-competitive type and highly specific (Nordenfelt *et al* 1980). We now report on the effect of PFA and PAA on the DNA polymerase associated with WHV.

One ml samples of serum specimens for DNA polymerase assay were prepared as described previously (Nordenfelt & Kjellen 1975). The sera were taken from woodchucks known to be carriers of WHV. Electron microscopy on one of the pellets used in the assay showed a large number of WHV particles. Assay conditions were as described previously (Kaplan *et al* 1973).

Members of a novel class of viruses. Like HBV, WHV has been shown to be associated with both chronic hepatitis and hepatocellular carcinoma (Summers *et al* 1978). Therefore woodchucks are a possible model

according to the method of Nylen (1924). The inhibitors were added to the reaction mixtures at 4 °C prior to incubating for 3 h at 37 °C. Assays were carried out in duplicate.

THE OCCURRENCE OF THE TREHALOSE FERMENTING, TETRACYCLINE AND POLYMYXIN RESISTANT PHENOTYPE AMONG THE *ENTEROBACTERIACEÆ*

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Siboni K The occurrence of the trehalose fermenting tetracycline and polymyxin resistant phenotype among the *Enterobacteriaceæ* Acta path microbiol scand Sect B 88 185-188 1980

In *Proteus morganii*, *P. mirabilis* and *Providencia stuartii* the ability to ferment trehalose and resistance to tetracycline were associated in 90%-97% of the strains. The same was true of at least 78% of the strains of *Serratia marcescens*. *Proteus vulgaris* showed a more quantitative association of the two traits. As the characters occur independently in 3-10% of the strains the association is considered to be due to simultaneous selection in some natural niche. The trehalose fermenting tetracycline and polymyxin resistant species ferment few other carbohydrates fewer than the remainder of the *Serratia* species.

Key words: *Proteus*, *Providencia*, *Serratia*, polymyxin resistance, tetracycline resistance, trehalose fermentation.

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A previous paper (Siboni 1976) was devoted to the description of the correlation of trehalose fermentation (tr+) and resistance to tetracycline (T-R) in *Proteus morganii*. It was concluded that *P. morganii* could be divided into two natural groups. A consisting of tr-, T-S strains, and B of tr+, T-R strains as the combinations tr-T-R and tr+, T-S were rare.

The present study has concentrated on a description of the occurrence of the tr+ T-R phenotype among *Enterobacteriaceæ*.

MATERIALS AND METHODS

The strains were collected from patients specimens in the period 1975-78. Media and sensitivity testing were as described by Siboni (1976). For the diffusion method was used the medium described by Casals & Pedersen (1978). Fermentation media were read 1-7 days after inoculation and the strains were identified according to Edwards & Ewing (1972) and to Grimont & (1977). The reactions used for identification of swarming strains of *Proteus mirabilis* and *Proteus vulgaris* were production of indol -/+ presence of ornithine decarboxylase +/- fermentation of lactose -/+ maltose -/+ and trehalose +/late and liquefaction of gelatin +/-.

Tr- mutants of tr+ *P. morganii* were isolated as described earlier (Siboni 1976).

The correlation between results from testing sensu

The DNA polymerase activity in the presence of inhibitor as determined by the incorporation of ^{32}P -TTP into acid precipitable radioactivity was compared to the incorporation without inhibitor. As shown in Table 1, PFA inhibits WHV associated DNA polymerase activity to approximately 80% at a concentration of 500 μM and about 60% at 100 μM . This is a clear inhibitory effect but less than the effect on the HBV polymerase which is inhibited to 90% by 10 μM . PAA has less effect. The results in these experiments vary with a difference between 0 to 50% inhibitory effect; however, the mean of about 26% is in complete accordance with the effect on the HBV-associated DNA polymerase.

The inhibitory effect of PFA on HBV and WHV associated DNA polymerases appears to be very specific. For instance, a 50% inhibition of cellular DNA synthesis has been seen only at a concentration of 2000 μM PFA (Stenberg & Larsson 1978) and cellular DNA

polymerase γ is not inhibited by 500 μM (Eriksson, personal communication). No inhibition of bacterial or mycoplasma growth by PFA has been observed in 16 μM (unpubl. obs). Furthermore, the inhibition by PFA but not by PAA discriminates these polymerases from several other polymerases tested (Helgstrand *et al.* 1978; Reno *et al.* 1978). It has therefore been suggested that PFA

test
al.
polymerase

Additionally, it is known that PFA inhibits herpetic simplex DNA polymerase *in vitro* and has a therapeutic activity in animals infected with herpes virus (Nilsson *et al.* 1978). If the HBV and WHV DNA polymerases are necessary for viral replication, it is possible that inhibiting them PFA could have a therapeutic effect. Studies have been initiated to test this hypothesis.

TABLE 1. Inhibition of WHV DNA Polymerase Activity by PFA and PAA in Serum Preparation from 4 Mice

Animal	Incorporation in cpm in the absence of PFA or PAA	500 μM PFA percentage inhib	100 μM PFA percentage inhib	10 μM PFA percentage inhib	500 μM PAA percentage inhib
1	234		47	26	
	1920	79	56		0
2	42526	88	73	40	
3	20344	89			57
4	10281		71	28	21

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THE OCCURRENCE OF THE TREHALOSE FERMENTING, TETRACYCLINE AND POLYMYXIN RESISTANT PHENOTYPE AMONG THE *ENTEROBACTERIACEÆ*

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Key words: *Proteus*, *Providencia*, *Serratia*, polymyxin resistance, tetracycline resistance, trehalose fermentation.

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A previous paper (Siboni 1976) was devoted to the description of the correlation of trehalose fermentation (tr+) and resistance to tetracycline (T R) in *Proteus morganii*. It was concluded that *P. morganii* could be divided into two natural groups. A consisting of tr- T S strains and B of tr+ T R strains as the combinations tr T R and tr+ T S were rare.

The present study has concentrated on a description of the occurrence of the tr+ T R phenotype among *Enterobacteriaceæ* naturally resistant to polymyxin, namely other *Proteus* species, *Providencia* and *Serratia*. It will be discussed how tr+ T R species differ from tr- T S and tr- T R species.

MATERIALS AND METHODS

The strains were collected from patients' specimens in the period 1975-78. Media and sensitivity testing were as described by Siboni (1976). For the diffusion method was used the medium described by Casals & Pedersen (1978). Fermentation media were read 1-7 days after inoculation and the strains were identified according to Edwards & Ewing (1961).

For the identification of indole -/+ presence of ornithine decarboxylase +/- fermentation of lactose -/+ maltose +/- and trehalose +/- and liquefaction of gelatin +/-.

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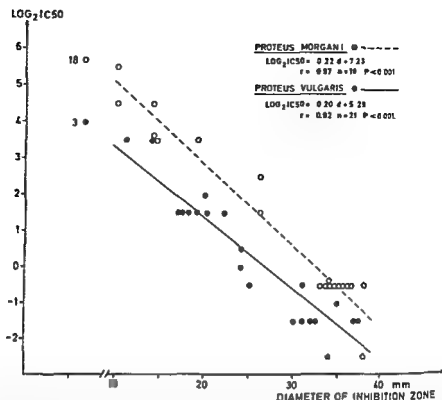


Fig 1 Sensitivity to tetracycline of selected strains of *P. morganii* and *P. vulgaris*. IC_{50} is correlated to the diameter of the inhibition zone. The same inhibition zone corresponds to a lower IC_{50} with *P. vulgaris* the difference is an average of 1.31 \log_2 (2.48 times).

sensitivity to tetracycline of strains of *P. morganii* and *P. vulgaris* by the diffusion method and by the tube dilution method is shown in Fig 1.

The strains of *Serratia* and their sensitivity to tetracycline have been described elsewhere (Siboni 1980).

RESULTS

P. morganii. To the strains collected in 1975 (Siboni 1976) have been added 131 strains from 1976. The distribution (Table 1) is still incompatible with a

TABLE 1 Strains of *Proteus morganii* Distributed According to Fermentation of Trehalose and Sensitivity to Tetracycline

	Trehalose fermenter	Trehalose non fermenter	Totals
Tetracycline resistant	18	21	39
Tetracycline sensitive	2	182	184
Totals	20	203	223

TABLE 2 Strains of *Proteus mirabilis* Distributed According to Fermentation of Trehalose and Sensitivity to Tetracycline

	Trehalose fermenter	Trehalose non fermenter	Totals
Tetracycline resistant	249	5	254
Tetracycline sensitive	2	0	2
Totals	251	5	256

random distribution according to the two criteria tr^+/tr^- and $\text{T R}/\text{T S}$ ($P < 10^{-13}$) (Fisher 1971) and the association $\text{tr}^+ \text{T R}$ or $\text{tr}^- \text{T S}$ exist in 0.90 of the strains.

Table 1 shows that $\text{tr}^+ \text{T S}$ strains do exist. These strains $\text{tr}^- \text{T S}$ mutants could be isolated. Eight of the 39 T R strains were resistant to chloramphenicol.

P. mirabilis. The distribution in Table 2 is compatible with a random distribution according to the two criteria ($P > 0.05$) i.e.

LE 3 Strains of *Proteus vulgaris* Distributed According to Fermentation of Trehalose and Sensitivity to Tetracycline

Sensitivity to tetracycline (diameter of inhibition zone)	Number of days till fermentation of trehalose							Totals
	1	2	3	4	5	6	7	
≤ 14 mm	6	1	2	—	—	—	—	9
15-22 mm	6	22	2	—	1	1	—	32
23-27 mm	2	1	3	1	1	—	—	8
≥ 28 mm	—	5	11	5	2	1	1	25
Totals	14	29	18	6	4	2	1	74

Correlation coefficient, $r = 0.5308$, $r^2 = 0.100$, $t = 5.3$, $P < 0.001$

fermentation of trehalose was associated with resistance to tetracycline in $\frac{249}{256} = 0.97$ of the strains.

P. vulgaris. There was not the qualitative difference between the tr^+/tr^- and T^R/T^S strains observed with *P. morganii* and *P. mirabilis*. All the strains fermented trehalose sooner or later, but the quicker the fermentation, the more resistant to tetracycline were the strains. The figures in Table 3 give a correlation coefficient r of 0.53 between the number of days till fermentation of trehalose and the diameter of the inhibition zone with tetracycline. *P. rettgeri*. All 17 strains were tr^- and T^R , so the two characters were not associated.

Providencia stuartii. All strains were tr^+ . 22 were T^R , so the two characters were associated in $\frac{22}{22} = 0.92$.

17 of the strains were resistant to chloramphenicol.

Serratia marcescens. All 56 strains were tr^+ . 51 were T^R , so the characters were associated in $\frac{51}{56} = 0.91$. Even if the 33 multiresistant strains with suspected R factors (Siboni 1980) are excluded, the characters are associated in $\frac{48}{53} = 0.78$.

S. liquefaciens. The 24 strains were tr^+ and T^S , so the two characters were not associated. The same was true of *S. plymouthica* (*S. kishinensis*) 6 strains and with *S. marinorubra* (*S. rubidica*) 10 strains.

All strains of the nine species were resistant to polymyxin B 10 µg/ml, with the exception of 4 strains of *P. morganii*, 3 strains of *P. mirabilis*, 5 strains of *S. plymouthica* and 2 strains of *S. marinorubra*.

DISCUSSION

Four species *P. morganii*, *P. mirabilis*, *Providencia stuartii* and *S. marcescens* showed association of fermentation of trehalose and resistance to tetracycline in (78) 90-97% of the strains. The few strains which were $tr^+ T^S$ or $tr^- T^R$ show that the two characters are not dependent on one another. This is also shown by the fact that tr^- mutants could be selected from tr^+ strains of *P. morganii* without changing the resistance to tetracycline (Siboni 1976).

The association of the two characters is therefore considered to be caused by simultaneous selection in a niche where the strains may ferment trehalose if they are resistant to tetracycline, e.g. a niche inhabited by *Streptomyces* species which are able to synthesise trehalose (Elbein 1967) and produce tetracycline and chloramphenicol. This could explain why so many strains of *Providencia stuartii* and of the T^R *P. morganii* are resistant to chloramphenicol. As chloramphenicol inhibits tetracycline inactivating enzymes (Hofmeister 1970), chloramphenicol resistance in a strain would add to its resistance to tetracycline in the presence of both tetracycline and chloramphenicol.

This hypothesis is supported by the large $tr^- T^S$ and chloramphenicol sensitive group of *P. morganii* (Siboni's group A, 1976) and by the rarity of the $tr^+ T^S$ and $tr^- T^R$ strains of this species. When one of the characters has been lost, selection is against the other one. *P. vulgaris* also demonstrated the correlation of trehalose fermentation to resistance to tetracycline (Table 3) but quantitatively as

with the species mentioned above, the correlation was not absolute the correlation coefficient was low, $r = 0.53$, but definitely $\neq 0$ ($r_s = 0.01$, $t = 5.3$, $f = 72$, $P < 0.001$).

The correlation $\text{tr}^{+}/\text{T-R}$ was not found in *S. liquefaciens*, *S. plymouthica* and *S. marinorubra*. The explanation may be that these species are not so restricted in their carbohydrate sources as are *P. morganii*, *P. vulgaris*, *P. mirabilis*, *Providencia stuartii* and *Serratia marcescens*. While the former species ferment five or six dihexoses and monopen-toses, the latter ferment less than four.

The grouping of *S. marcescens* with regard to the traits discussed tr⁺, T-R and polymyxin resistant, with *Providencia stuartii*, *P. mirabilis*, *P. vulgaris* and the tr⁺ group of *P.morganii* (Siboni's group B 1976) is not so surprising. Its DNA is only slightly related to that of *Proteus* (Brenner *et al* 1978) but its overall similarity to *Proteus* is at the 75% level (Johnson *et al* 1975) i.e. at the same level as to *S. liquefaciens* and to *S. phymouthica* and higher than the similarity to *S. marnorubra* 70% (Grimont *et al* 1977). The species mentioned have not been compared in one single investigation.

The niche hypothesis formulated above and to some extent substantiated does not cover the tr-T-R *Proteus reitteri*. Examination of sewage and soil has not so far disclosed the niche suited for the trehalose fermenting, tetracycline and polymyxin resistant species.

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SENSITIVITY OF *SERRATIA* TO TETRACYCLINE

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Siboni K. Sensitivity of *Serratia* to tetracycline Acta path microbiol scand Sect B 88 189-192 1980

S. marcescens had two levels of resistance to tetracycline bacteriostatic end points 32 and 180 µg/ml the latter group consisted of strains resistant to carbenicillin and to streptomycin *S. plymouthica* *S. liquefaciens* and *S. marinorubra* were sensitive to tetracycline with the last named as the least sensitive species Less difference was found between the bactericidal end points of the four species but there was still 1-2 two fold steps between *S. marcescens* and the remaining three species

Key words *Serratia* tetracycline sensitivity

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Different investigators have found 76-86 per cent of strains of *Serratia marcescens* resistant to tetracycline (Wilfert et al 1970 85% had MIC > 5 µg/ml Greenup & Blazevic 1971 76% had MIC > 25 µg/ml Grimont et al 1977 84% had MIC > 25 µg/ml 98% had MIC > 5 µg/ml) Federos & O'Brien (1969) found two levels of resistance to tetracycline MIC ~ 500 µg/ml and MIC ~ 31-62 µg/ml the strains in the former group were also resistant to sulphonamide streptomycin and chloramphenicol and they were highly resistant to ampicillin MIC ≥ 250 µg/ml these resistance traits were transferable to *E. coli* F Farrar & O'Dell (1976) examined a few strains and showed that the multiresistant strains with high ampicillin resistance were resistant to carbenicillin (MIC > 300 µg/ml) while the oligoresistant strains of low ampicillin and tetracycline resistance were sensitive to carbenicillin (MIC ≤ 6 µg/ml) Greenup & Blazevic (1971) found 15 of their 17 *Serratia liquefaciens* strains sensitive to tetracycline 125 µg/ml all the strains were sensitive to streptomycin to chloramphenicol and to carbenicillin Twenty two strains of *Serratia marinorubra* were resistant to tetracycline 5 µg/ml sensitive to 25 µg/ml (1977) The same

investigators found 20 strains of *Serratia plymouthica* sensitive to tetracycline 5 µg/ml

It has been the purpose of the present investigation to describe the occurrence of *Serratia* in patients specimens the level of resistance to tetracycline in the different species and the correlation of this resistance to other resistance traits

MATERIALS AND METHODS

The strains of *S. marcescens* and *S. liquefaciens* were isolated from 77 100 specimens received in 1976 and 1977 in the Regional Laboratory of Statens Seruminstitut at Odense University Hospital from hospitals and general practitioners in the county of Funen which has 500 000 inhabitants The strains were isolated on modified Conradi Drigalski agar and identified in

been used

Only three strains of *S. marinorubra* (*S. rubidexa*) and one of *S. plymouthica* (*S. kilensis*) were isolated in 1976 and 1977

In the study were therefore included *S. marinorubra* Four strains isolated earlier and three strains received from Dr Hans Lautrop Statens Seruminstitut

Copenhagen *S. plymouthica* Five strains received from Dr Hans Lautrop Sensitivity tests Routine diffusion tests were performed using Rosco's tablets (neosensitabs® Rosco DK 2630 Taastrup) on Danish blood agar Statens Serum Institut as described by Casals & Pedersen (1978) Strains showing inhibition zone diameters > 23 mm corresponding to MIC < 5 µg/ml tetracycline < 20 µg/ml streptomycin < 15 µg/ml chloramphenicol < 15 µg/ml sulphonamide and < 10 µg/ml carbenicillin were regarded as sensitive Determination of IC50 in two fold tube dilution tests and other media were as described by Siboni (1976) $IC_{50} \times \sqrt{2} = MIC$

The resistance types of *S. marcescens* determined in diffusion tests are recorded in Table 1 At least four strains of each of the resistance types carbenicillin resistant/tetracycline resistant carbenicillin sensitive/tetracycline resistant and carbenicillin sensitive/tetracycline sensitive of *S. marcescens* and of each of the remaining three *Serratia* species were tested by the tube dilution method (Table 2)

The bactericidal concentration was determined as the lowest concentration which killed 99% of the bacterial cells after 18 h of incubation of the broth dilution series This definition was feasible with the small inoculum used average $10^{4.4}$ cfu (Barry & Sabath 1974)

Wilcoxon's non parametric test was used to compare the end points of the different species and groups (Wilcoxon 1945)

RESULTS

S. marcescens (56 strains) Table 1 shows that strains resistant to carbenicillin were also resistant to tetracycline and to streptomycin and some of them to chloramphenicol while these resistance

traits were rarer among the strains sensitive to carbenicillin, none of the latter strains was resistant to chloramphenicol The bacteriostatic end points with tetracycline were higher in the group resistant to carbenicillin than in the carbenicillin sensitive group ($2\alpha < 0.01$) (Table 2) The bactericidal end points of the two groups differed less 90.5–25 µg/ml ($2\alpha < 0.02$)

Five strains of the carbenicillin sensitive group were sensitive to tetracycline in routine diffusion tests Tube dilution tests with these strains gave $IC_{50} \leq 2^{2.5} = 5.7$ µg/ml (Table 2)

S. liquefaciens (24 strains) and *S. plymouthica* (strains) All strains were sensitive to tetracycline in diffusion tests and ten and four strains respectively in tube dilution tests (bacteriostatic end points) *S. plymouthica* was even more sensitive than *S. liquefaciens* (Table 2)

S. marzinorubra (10 strains) was sensitive to tetracycline in diffusion tests but the tube dilution test gave a borderline value $IC_{50} = 2^{2.55} = 4$ µg/ml in agreement with Grimont *et al.* (1976) (Table 2)

The bactericidal end points of the last three species were the same (Table 2)

DISCUSSION

Bacteriostatic end points *S. marcescens* had 1 level of resistance to tetracycline IC_{50} 32 and 1 µg/ml The high level resistance was almost exclusively found in strains resistant to carbenicillin (Table 2) Table 1 shows that this group consisted

TABLE 1 *Strains of Serratia marcescens* Distributed According to Carbenicillin Resistance and to Other Resistance Types (Diffusion Method)

Resistance type	Carbenicillin resistant		Carbenicillin sensitive		Totals
	observed	expected	observed	expected	
SuTSC	7	0.70	—	1.25	7
SuTS	13	4.85	8	8.75	21
TS	—	—	5	9.32	5
SuT	—	14.45	1	6.97	1
T	—	—	17	6.49	17
O	—	—	5	3.21	5
Totals	20	20.00	36	35.99	56

Su T S and C resistant to sulphonamide tetracycline streptomycin and chloramphenicol
O sensitive to the named antimicrobials

The expected values have been calculated from products of the frequencies of the single resistance traits expected if the traits occurred independently
(Example $p(\text{SuTSC}) = 29/56 \times 51/56 \times 33/56 \times 7/56 = 0.035$)

TABLE 2. Sensitivity to Tetracycline of Selected Strains of *Serratia* with the Tube Dilution Method

\log_2 IC50	-1.5	-1.0	-0.5	0.0	0.5	1.0	2.0	2.8	4.0	5.7	8.0	16.0	32.0	64.0	128	256	\log_2 geometric mean
IC50 $\mu\text{g/ml}$	0.5																
<i>S. marcescens</i>																	
carbenicillin R																	7.38
tetracycline R																	7.54
bacteriostatic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
bactericidal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
carbenicillin S																	
tetracycline R																	5.27
bacteriostatic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	7.07
bactericidal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
carbenicillin S																	
tetracycline S																	1.75
bacteriostatic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.50
bactericidal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	
<i>S. liquefaciens</i>																	
bacteriostatic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0.90
bactericidal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.90
<i>S. plymouhica</i>																	
bacteriostatic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-1.00
bactericidal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5.75
<i>S. marinarubra</i>																	
bacteriostatic	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2.55
bactericidal	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	6.35

R resistant and S sensitive with the diffusion method

multiresistant strains in much higher numbers than could be expected if the resistance traits occurred independently ($P < 10^{-9}$) (Table 1 column expected). This agrees with the findings of Medeiros & O'Brien (1968), Farrar & O'Dell (1976) and Søgaard (1979) for *E. coli*. The carbenicillin sensitive group was dominated by strains resistant only to tetracycline and mainly of low level resistance (Table 2).

The two levels of resistance to tetracycline are implicit in the results of Greenup & Blazevic (1971).

The remaining three species were sensitive to tetracycline with *S. marinorubra* as the most resistant but they were more sensitive than the low level tetracycline resistant group of *S. marcescens* ($2\alpha < 0.01$). Bactericidal end points. The difference between the species was less than with the bacteriostatic end points, amounting at most to fourfold. The end points of the carbenicillin sensitive group of *S. marcescens* were lower than those of the carbenicillin resistant group ($2\alpha < 0.02$) and the end points of the three remaining species were lower than those of *S. marcescens* as a whole ($2\alpha < 0.01$).

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ANTIBIOTIC SENSITIVITY OF *HAEMOPHILUS INFLUENZAE* STRAINS INCLUDING THREE RECENT CHLORAMPHENICOL-RESISTANT ISOLATES

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The antibiotic sensitivity of 100 recent isolates of *Haemophilus influenzae* was determined. Three strains were resistant to chloramphenicol with minimal inhibitory concentrations of 18 µg/ml. Of these three resistant strains one produced β -lactamase and one was resistant to sulfamethoxazole-trimethoprim. The remaining strains were inhibited by 0.25-2.0 µg/ml of chloramphenicol. Ampicillin and benzylpenicillin were found to inhibit all but the β -lactamase producing strains at low concentrations. Regarding sulfamethoxazole-trimethoprim 96% had minimal inhibitory concentrations of 2.5-0.12 µg/ml or less while two strains were resistant. The *in vitro* efficacy of erythromycin against *H. influenzae* was low. The majority of the strains was inhibited by low concentrations of doxycycline and cefuroxime while cefoxitin exhibited minimal inhibitory concentrations values usually exceeding 1 µg/ml. The minimal inhibitory concentrations registered are compared to the concentrations of the different antibiotics attainable in certain body fluids.

Key words: *Haemophilus influenzae*, antibiotic sensitivity, chloramphenicol resistance.

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There are some previous reports on chloramphenicol resistant strains of *H. influenzae* with minimal inhibitory concentrations (MICs) varying from 8 to 50 µg/ml (4, 7, 10, 14, 20, 24, 25, 29, 32, 39). In this laboratory we have recently isolated three strains of *H. influenzae* resistant to chloramphenicol and to the best of our knowledge we are not aware of previous Scandinavian reports on chloramphenicol resistant *H. influenzae* strains.

These facts prompted us to test the susceptibility of 100 recent clinical isolates of *H. influenzae* to the antibiotics most frequently used when treating *H. influenzae* infections.

MATERIALS AND METHODS

Bacterial strains. The 100 strains of *H. influenzae* tested were all recent clinical isolates. Three strains originated from blood cultures, 3 from conjunctival secretions, 3

from middle ear exudates, 1 from maxillary sinus, 1 from wound and 87 from patients with upper respiratory tract diseases. Only one strain from each patient was tested. The strains were identified by culture characteristics and growth dependence of X and V factors. The organisms were stored at -26 °C for three months to 1 week in nutrient broth containing horse serum and glucose.

Media. The antibiotic susceptibility tests were performed as broth microdilution tests described by Kirven & Thorsberry (21). Mueller Hinton broth pH 7.0 (Oxoid CM 405) was supplemented with 0.2% Hemin (H896 Bovin/Equin, Sigma) and 0.5% nicotinamide adenine dinucleotide (Sigma D 7381) (M-H broth). The MIC 2000 dispenser (Dynatech Laboratories, Alexandria, VA, USA) was used for dispensing 100 µl amounts of broth containing antibiotics into the wells of microdilution plates. The plates were stored at -26 °C for a maximum period of 3 weeks prior to use. The antibiotic concentrations of the solutions dispensed were checked both by conventional microbiological assays (11) and by observing the MICs of bacterial strains with known

susceptibility *E. coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923 and *Streptococcus faecalis* ATCC 29212. The chloramphenicol MICs of the resistant strains were performed in tubes containing 1 ml of broth.

Antibiotics All antibiotics were of known potency and were used in twofold dilutions.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) The strains were cultured in supplemented M II broth for 24 hours and were diluted to contain about 5×10^7 CFU/ml. The microdilution plates were inoculated with the Dynatech MIC 2000 inoculator and each well received approximately 10^4 CFU. The microdilution plates were incubated for 18 hours at 36°C in 5% CO_2 atmosphere. The MICs were read as the lowest concentration of drugs that gave no visible growth. For MBC determinations the Dynatech inoculator was used for transferring inoculum from each well to chocolate agar plates. The MBCs were read after 24 hours incubation at 36°C in 5% CO_2 atmosphere as the lowest concentration giving no growth.

Beta-lactamase test Beta lactamase production was detected by the chromogenic cephalosporin (30).

Enzyme production Inactivation of chloramphenicol by the chloramphenicol resistant strains was demonstrated by the technique of Slack *et al* (36). A chocolate agar plate was seeded with a chloramphenicol sensitive *E. coli*. Colonies from the chloramphenicol resistant *H. influenzae* strains were applied to filterpaper discs, one disc for each strain. A chloramphenicol sensitive *H. influenzae* strain used as control was applied to the surface of two other filterpaper discs. After about five minutes a 30 µg chloramphenicol disc was placed on the top of each disc except on one of the discs containing the sensitive *H. influenzae* strain. The plate was read after incubation for 18 hours at 37°C .

RESULTS

The results of the susceptibility tests are presented in Fig. 1 A-H and Table 1.

Fig. 1 MIC — and MBC — of 100 strains of *Haemophilus influenzae* expressed as cumulative percentages of strains inhibited with increasing drug concentration (A) erythromycin, (B) ampicillin, (C) doxycycline, (D) chloramphenicol, (E) cefuroxime, (F) cefoxitin, (G) sulfamethoxazole, trimethoprim, (H) benzylpenicillin.

Erythromycin The MICs of 38% of the strains exceeded 5 µg/ml. The remaining strains were inhibited by concentrations from 1.25 to 5.0 µg/ml with median MIC 4.37 µg/ml. MBC was >5 µg/ml for 68% and 2.5–5.0 µg/ml for the remaining strains.

Ampicillin Two strains were beta-lactamase producing with MICs more than 5 µg/ml. The majority of the strains had MICs between 0.31 and 0.62 µg/ml with median MIC of 0.34 µg/ml. MBCs were identical to the MICs for all except strains that had MBCs one twofold concentration higher.

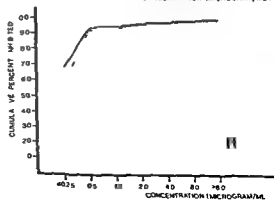
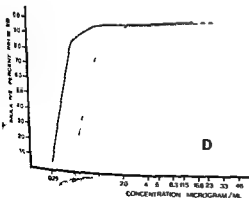
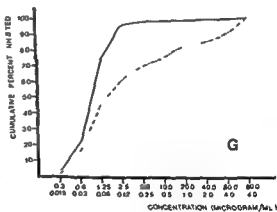
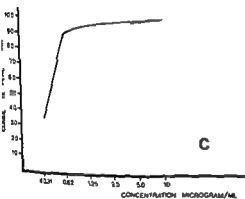
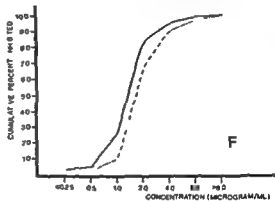
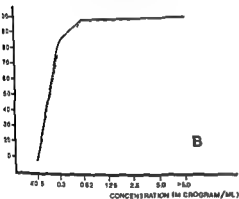
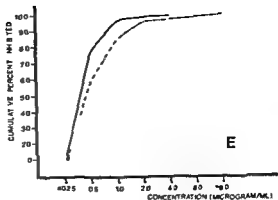
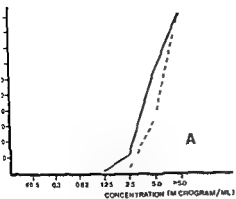
Doxycycline The MICs of 35% of the strains were less than 0.31 µg/ml while 63 had MICs from 0.62 to 2.5 µg/ml. Two strains were resistant with MICs 5.0 and 10.0 µg/ml. Almost all strains had MBCs a dilution higher than their MICs.

Chloramphenicol All strains except the resistant isolates were inhibited by 0.25–2.0 µg/ml, mean 0.53 µg/ml. MBCs for the 97 sensitive strains were 0.5–4.0 µg/ml, mean 1.1 µg/ml.

Figure 2 demonstrates a zone of inhibition around the disc soaked with the sensitive strain but not around the filterpaper discs containing the resistant strains. MICs of the chloramphenicol resistant strains are presented in Table 1. The first isolate (No. 1) was a *H. influenzae* type b derived from the nasopharyngeal secretion of a 7 year old boy, earlier treated with penicillin for repeated episodes of otitis media.

TABLE 1. Antibiotic Sensitivity of the Chloramphenicol Resistant Strains

Strain	MIC µg/ml			MBC µg/ml		
	1	2	3	1	2	3
Erythromycin	5.0	>5.0	5.0	>5.0	>5.0	>5.0
Ampicillin	<0.15	>5.0	0.31	<0.15	>5.0	0.31
Doxycycline	1.25	1.25	2.5	1.25	1.25	2.5
Chloramphenicol	16.0	16.0	16.0	46.0	23.0	23.0
Cefuroxime	<0.25	0.5	0.5	<0.25	0.5	0.5
Cefoxitin	1.0	2.0	2.0	2.0	2.0	2.0
Cefoxitin	2.5	2.5	80.0	80.0	40.0	>80.0
Sulfamethoxazole/Trimethoprim	>10.12	10.12	14.0	14.0	12.0	14.0
Benzylpenicillin	<0.25	>8.0	0.5	<0.25	>8.0	0.5
Beta-lactamase	—	+	—	—	+	—



while the majority were inactivated by 0.5 µg/ml or less. The MBCs were mostly identical to the MIC

DISCUSSION

The recent observations that certain strains of *H. influenzae* have acquired resistance not only to ampicillin but also to chloramphenicol is of concern when treating serious infections caused by *H. influenzae*.

In the present study three strains with decreased susceptibility to chloramphenicol were registered. These strains were found to inactivate chloramphenicol probably as a result of enzymatic activity in accordance with other studies on chloramphenicol-resistant *H. influenzae* strains (22, 25). van Klingeren *et al.* have demonstrated that the chloramphenicol resistance of *H. influenzae* is plasmid mediated and probably transferred by conjugation (22).

The results registered in this study apart from chloramphenicol-resistant strains are well in accordance with previous reports on the susceptibility of *H. influenzae* to chloramphenicol (12, 14, 40, 41). In the present study 98% of the strains had MICs when tested against SXT. This is in sharp contrast to the figures presented by May & Davison who demonstrated 55% resistance to SXT employing a disk diffusion method (26). A recent report demonstrates a correlation between *H. influenzae* strains with high MBCs to SXT and persistence of strains with this ability from the nasopharynx of children (21, 42). In the present study a considerable portion of the strains is shown to have a high MBC despite a low MIC.

It has recently been demonstrated that ventricular concentration of chloramphenicol after doses of 10 mg/kg bodyweight every 6 hours in newborn children may be unpredictable and so low as to jeopardize treatment of *H. influenzae* strains with MICs of 4–8 µg/ml (43). This is a most likely explanation for treatment failures reported in children. However, the doses used in children above 1 month old and adults ought to result in sufficient cerebrospinal concentrations for chloramphenicol-sensitive strains (38).

Regarding ampicillin all strains in the present study with the exception of the beta-lactamase-producing strains were inhibited by 0.62 µg/ml. These findings are well in accordance with earlier reports (4, 14, 19). Comparison between concentrations measured in cerebrospinal fluid and ear exudates indicate that ampicillin levels in the body fluids are well above the MICs of non-beta-lactamase-producing strains of *H. influenzae* (16, 31, 32). Sufficient!

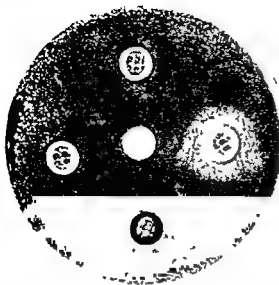


Fig. 2. Demonstration of chloramphenicol inactivation. In the middle: Control sensitive strain (no chloramphenicol disk applied). To the right: Chloramphenicol-sensitive strain. The remaining disks: The three chloramphenicol-inactivating strains.

The second chloramphenicol-resistant strain (No. 2) was isolated from the epipharyngeal culture of a 1-year-old girl with recurrent otitis media, treated in the past with penicillin. This strain produced beta-lactamase and was nontypable.

The third chloramphenicol-resistant strain (No. 3) was nontypable and was isolated from a chronic neck fistula of a 18-year-old woman. The strain was also resistant to sulfamethoxazole-trimethoprim. Two months earlier exudate from the fistula had grown a *H. influenzae* strain sensitive to both SXT and chloramphenicol.

The three chloramphenicol-resistant strains had moderately reduced sensitivity to doxycycline.

Cefuroxime. The majority of the strains had MICs of 0.5 µg/ml or less, while 3% were less sensitive with MICs of 2.0–4.0 µg/ml. The beta-lactamase-producing strains had both MICs and MBCs of 0.5 µg/ml for cefuroxime.

Cefoxitin. Fig. 1F demonstrated that three of the strains were inhibited by less than 0.25 µg/ml. One strain had MIC higher than 8.0 µg/ml. The remainder had MICs from 0.5 to 8.0 µg/ml, median value 1.93 µg/ml. The MBCs were mostly identical to the MICs. A few had MBCs a dilution step higher than their MICs.

Sulfamethoxazole-Trimethoprim. The MICs of 96% of the strains were 2.5/0.12 µg/ml or less, while 2 strains had MICs exceeding 8.0/0.40 µg/ml. Despite low MICs several strains demonstrated high MBCs.

Benzylpenicillin. Four strains had MICs of 1.0–4.0 µg/ml and two were beta-lactamase-producing

med in sinus mucosa when treating sinusitis

concentration of both sulfamethoxazole and trimethoprim to cerebrospinal fluid and other body fluid is 1:1 (13, 34-37). Thus a combination of these drugs could be an alternative in treating *H. influenzae* meningitis and other serious infections caused by this organism. Clinical experience regarding treatment of meningitis is limited but the combination has been used with success in certain cases (34). The finding that 2% of the strains had MICs in excess of levels obtained in CFS is of some concern.

The sensitivity to cefuroxime and cefoxitin was in agreement with earlier findings (5-27). There are a few reports on cefuroxime treatment of meningitis (1, 33). Renlund & Pettay demonstrated concentrations of cefuroxime in cerebrospinal fluid from 2-3-1 µg/ml in patients with inflamed meninges (33).

cefuroxime (28).

About one third of the strains had MICs exceeding 5 µg/ml when tested against erythromycin. There are conflicting reports on the efficiency of erythromycin against *H. influenzae*. Kirven & Hornsberry (21) and Kalm *et al* (18) found the majority of the *H. influenzae* strains to be inhibited by 4 µg/ml. On the other hand, ...

inhibited by 0.5 µg/ml or less. Self reported that the majority of typable and nontypable *H. influenzae* strains were inhibited by 3-12 µg/ml while the typeable strains were more sensitive to erythromycin (35). These differences might be explained by the fact that the activity of erythromycin is affected by changes in pH. Further the pH-dependence of erythromycin makes evaluation of concentrations in body fluids difficult since the pH in certain purulent inflammation processes deviates from normal as demonstrated by Carenfelt & Lundberg (8). Bass *et al* found that the concentrations of erythromycin succinate in middle ear fluid were 0.07-1.02 µg/ml which in view of our observations seem to be unsatisfactory levels (6). Further in treatment of otitis media caused by *H. influenzae* Howie & Ploussard observed treatment failure when only erythromycin was used (15). Kalm *et al* demonstrated concentrations of erythromycin stearate in sinus secretion ranging from 0.3 to 2.5 µg/ml when the orally given dose was 500 mg 3 times a day while the measured concentrations in sinus secretion were 0.2-1.1 µg/ml when 500 mg twice a day was administered. The clinical results were better with

the higher dose but the gastrointestinal effects were more common (18).

The results regarding doxycycline are well in accordance with earlier reports (4, 21). The majority of the strains tested had MICs below the concentrations of doxycycline demonstrated in sinus secretions and in different tissues including lungs and pleura (3-23).

Benzylpenicillin was highly active in vitro against *H. influenzae* strains. Our results differ slightly from those reported by Kamme (19) and Barret *et al* (4) using agar dilution method. The efficacy in vivo in certain cases of pneumonia caused by *H. influenzae* was demonstrated by Asmar *et al* treating children with benzylpenicillin (2).

It is becoming increasingly obvious that the treatment of severe *H. influenzae* infections has to be guided by proper sensitivity testing as the sensitivity pattern of some strains is unpredictable.

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CHANGES IN VIRULENCE, M PROTEIN AND IgG Fc RECEPTOR ACTIVITY IN A TYPE 12 GROUP A STREPTOCOCCAL STRAIN DURING MOUSE PASSAGES

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A type 12 group A strain (1800) was passaged serially through mice 25 times. The ability to survive in normal human blood dropped from a growth index of 52 after the first passage to 1 after four passages. After 14 passages the growth index increased again and stabilized above 30. The virulence for mice increased from a LD₅₀ of 10³ colony forming units (CFU) to 10-100 CFU after 7 passages and then remained constant. The M12 antigen disappeared after 4 passages as tested by immunodiffusion, electroimmunoassay and indirect bactericidal tests. Three antisera raised in rabbits against strains originally belonging to types M13, M12 and M46 but devoid of type antigens after mouse passages showed high bactericidal indices against the 1800 strain after 14 or more passages on mice. Anti type M1 serum was also found bactericidal for the passaged strains. The IgG Fc receptor activity of the strain isolated after each mouse passage was tested in hemagglutination experiments with human red blood cells coated with «incomplete» anti Rh and hot hydrochloric acid extracts of the strains. The capacity to agglutinate «Rupley»-coated cells increased gradually during the first 12 passages and subsequently the titres of the extracts stabilized between 1:160 and 1:320. The HUN coat useful for detection of the G3m (S) marker gave titres increasing with the number of passages while the titres for IgG1 coats kept at 1:4 or below. On background of these results the possible role of the IgG Fc-receptor as a virulence factor is discussed.

Key words: Streptococci, M protein, bactericidal test, human IgG Fc, anti human Ig, Gm markers.

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The laboratory methods recommended for the investigation of the virulence of group A streptococci include examination of mouse virulence and test of the ability of the strain to grow in normal human blood (13, 14, 17, 25). The hyaluronic acid capsule - and in particular - the type specific antigens or M proteins are virulence factors of recognized importance (12, 20). It is a widespread opinion that a strain which grows in normal human blood and is virulent contains M protein

also when extracts of the strain fail to precipitate with type-specific antisera. Nevertheless, only type specific antibodies are generally assumed to promote phagocytosis in human blood. It is a matter of discussion whether group A streptococcal strains might be entirely devoid of M protein or substances closely resembling the M protein (9, 10). Hence the term «absence of M protein» refers to the functional absence of M protein as defined by the tests for virulence referred to above.

In addition to the M protein and the streptococcal capsule some other, hitherto unknown structures might be important for streptococcal virulence (1). Recently, it was found (5) that many group A streptococci have receptors for the Fc fragment of IgG the solubilized receptors display a variation in affinity between streptococcal types, e.g. with respect to reactivity for the human IgG1 and IgG3 subclasses (3). It remains to be investigated whether these receptors which are distinct from the M protein (4) could interfere with antibody-mediated phagocytosis.

One of the usual procedures for reestablishing the M protein production of a strain is to pass the bacteria serially through mice and simply follow the presumed acquisition of M antigen by testing these passaged strains for growth in normal human blood (13). The present investigation concerns the changes observed in type 12 group A streptococcal strain during serial mouse passages. In contrast to the concepts about M protein and virulence referred to above it was found that the type 12 antigen was lost during the passages although the virulence for mice and the ability to grow in human blood increased. During the passages the bacterial content of receptors for the Fc part of human IgG also increased.

MATERIALS AND METHODS

Bacterial Strains and Extracts

Group A streptococci type 12 (strain 1800 from the collection at the Institute of Hygiene and Epidemiology Prague) were used throughout the study. Hot hydrochloric acid extracts were prepared from over night cultures of the streptococci on 300 ml Todd Hewitt broth as previously described (3).

Mouse Passages of the M12 Strain

This method has been described previously (2). In brief 0.5 ml of over night culture of streptococci was injected intraperitoneally in white mice of 10–12 g. After each mouse had died from septicemia a suspension of the spleen was injected intraperitoneally in a new mouse etc. After each mouse passage the strain was isolated and designated according to the number of mouse passages performed before isolation e.g. the strain isolated after 15 passages was called M12/15.

The experiments described in this paper were confirmed in two repeated series of mouse passages with strain 1800.

Test of Streptococcal Strains for Mouse Virulence

White adult mice were inoculated intraperitoneally with 0.5 ml of tenfold dilutions 10^{-1} to 10^{-8} of an over night culture of the test strain. Five mice were injected with each suspension and the animals were observed for 3 days after which the number of deaths

was registered. The number of colony forming units (CFU) killing all mice was calculated (LD₁₀₀).

Rabbit Sera

Two rabbit antisera to type 12 group A streptococci were prepared: one in Leningrad other in Lund (Lu). Furthermore antiserum to type M strain 40/58 was prepared in Leningrad. These were used unabsorbed. Rabbit M typing sera against type 1 and type 12 group A streptococci from the Institute of Hygiene and Epidemiology Prague (Pi) were also used as was anti type 12 M typing serum kindly supplied by Dr El Kholy Cairo (Ca).

Antisera to three strains originally types 3, 12 and 4 but devoid of type specificity after hundreds of mouse passages (2) were also raised in rabbits. These sera were designated anti 3P, anti 12P and anti 46P respectively. The immunization schedule for the production of the and other antisera used followed the description given by Rott et al. (21).

The T typing sera were purchased from Chemap Prague.

Bactericidal Tests

These tests were performed essentially as described by Lancefield (13) and Šramek (25). In brief a measured quantity of streptococci (the inoculum) was mixed with freshly drawn human blood alone or in the presence of added serum and incubated for 3 hours in rotating tubes. The growth index (25) indicating the degree of inoculum propagation in normal human blood was calculated from the formula

$$\frac{\text{no. of CFU obtained with normal serum}}{\text{no. of CFU in inoculum}}$$

The bactericidal index (BI) indicates the degree of inoculum growth inhibition in the presence of anti-streptococcal serum as compared to controls with normal serum (24). This index was calculated from the formula

$$\frac{\text{no. of CFU obtained with normal serum}}{\text{no. of CFU obtained with antiserum}}$$

The scale used for expressing BI values was BI below 0 51–100 + 101–200 ++ 201–500 +++ above 500 ++++ (24).

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barbital buffer pH 8.6 containing 2 mM calcium ions was mixed with 0.8 ml anti type 12 serum. Lu: poured over a 205 x 110 mm glass plate. Circular wells taking 5 µl extract were cut 80 mm from the project anode. After application of the extract a current of 6 cm was applied for 16 h. The plate was stained with Coomassie Blue.

Streptococcal IgG Fc Receptors

se tests were performed essentially as described only (3). In brief Rh positive red blood cells were with «incomplete» anti Rh antibodies from individuals. The capacity of different dilutions of

subclass. Anti Rh KM and 317 are useful for ion of the human IgG1 genetic markers G1m(1) j1m(4), respectively. The anti Rh HUN detects the marker G1m(5).

Immunodiffusion in gel and precipitation test were performed as earlier (23).

Estimation of Protein

The protein content of the extracts was quantitated by modified Folin method (16).

RESULTS

Changes in the Virulence of the Streptococcal Strain during Mouse Passages

The growth index (which indicates the ability of a streptococcal strain to survive in human blood in test tubes) dropped from 52 after the first passage to one after four passages. The strains M12/5 to M12/13 also showed very low growth

indices (Fig. 1). After 14 passages the growth index increased again (see Table 1).

After each passage the isolated strains were also tested for mouse virulence. The results obtained show an increasing virulence during the first four passages: the LD₅₀ dropped from 10⁸ to 10³ CFU. During the following three passages the virulence was further increased. After the 7th passage the virulence stabilized and remained constant showing LD₅₀ between 10 and 100 CFU.

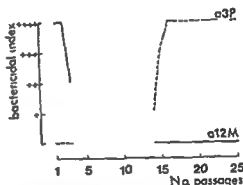
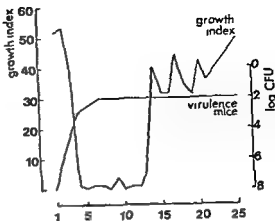


Fig. 1. Changes in virulence for mice and behaviour in bactericidal tests of a type 12 group A streptococcal strain during serial passages through mice. Abscissa: no. of passages performed. Ordinate: the virulence for mice given as the number of colony forming units (CFU) killing all of 5 mice.

index which indicates the capacity of a serum to kill the streptococci in the presence of normal human blood was calculated according to Stollerman *et al.* (24). a3P indicates the results with a rabbit antiserum to strain 3P and a12M the results with a rabbit antiserum to type M12 (the Le serum).

TABLE 1. Representative Bactericidal Tests with Some Selected Strains Obtained after Mouse Passages of Strain 1800

	Bactericidal test with strains					
	M12/1	M12/2	M12/3	M12/14	M12/19	M12/25
No. of CFU inoculated	105	93	93	55	86	106
Growth index	52	53	41	40	31	11
Bactericidal index						
anti-type 12 serum	>5488	1249	137	1	0	1
anti-3P serum	0	1	1	85	>2733	5168

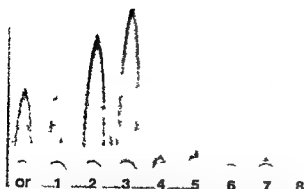


Fig 2 Electroimmunoassay with hot hydrochloric acid extracts of strain 1800 before and after passages through mice. Twenty ml agarose was mixed with 0.8 ml of the anti type 12 serum Lu and poured over a glass plate. Extracts were placed in the circular wells and after application of the current for 16 hours and washing the plate was stained with Coomassie Blue. Precipitates indicate the results of testing strains M12/1, M12/2, M12/3 etc. The extracts of strains M12/10–M12/25 not shown on the picture did not give any precipitates.

Loss of Type Specificity During the Mouse Passages

The Ca, Le and Lu anti type 12 sera were compared by immunodiffusion in experiments with the hydrochloric acid extracts of the strains isolated after each of the 25 passages. All three sera gave a strong precipitate against each of the extracts of M12/1, M12/2, M12/3 as well as the original strain. When the precipitates were compared with one another Ouchterlony type I reactions (interference with complete fusion) were obtained between all lines. This precipitate was not obtained with any of the extracts from the strains M12/4 to M12/25.

Figure 2 shows the results of electroimmunoassay quantitation of the M protein content in the hydrochloric acid extracts of the passed strain. The height of the rockets increased during the first three passages. However, the M protein precipitate disappeared and did not recur after the fourth passage.

The protein content in the hydrochloric acid extracts of the passed strains varied from 18 to 1 mg/ml. There was no correlation between the protein content of the extracts and the passages performed.

T typing (11) of the passed strains M12/1–7, 9, 10 and 25 was performed and all these strains were found to belong to T type 12. The other strains were spontaneously agglutinating and T typing could therefore not be performed.

The loss of M12 protein was also confirmed by the indirect bactericidal test using the anti type sera Le and Pr. The bactericidal index (which indicates the ability of an immune serum to kill streptococcal strain in the presence of fresh blood) was estimated as + + + + after two and + + after three. Because of the low growth index it was not possible to determine the bactericidal effect of the anti type 12 sera on strains M12/4 to M12/13. The strains M12/14 to M12/25 gave bactericidal indices with anti type 12 sera estimated as one (Fig. 1).

Recently a suitable donor for bactericidal test with strain M12/6 (growth index 53) was found (Havlicek personal communication). The strain was inhibited neither by anti M12 nor by anti M12 serum.

Test of the Passed Strains for Streptococcal IgG Fc Receptors

The extracts of the passed strains were tested for capacity to agglutinate R₁ sensitized human R₁.

TABLE 2 Agglutination of O Rh + Red cells Coated with Human IgG Anti Rh by Lancefield Extracts of Type 1 Group A Streptococci Subjected to Mouse Passage

Human IgG coat	Genetic marker detected by the coat	Titre of the extract of type 12 group A streptococci after mouse passage no						
		0	6	7	11	13	23	25
R ₁ (1:5)*		1:10	1:80	1:20	1:160	1:160	1:320	1:320
KM (1:5)*	G1m(1)	1:4	0	0	1:4	1:4	1:4	1:4
317 (1:5)*	G1m(4)	1:2	0	0	0	0	0	0
HUN (1:10)*	G3m(5)	1:10	1:10	1:10	1:20	1:20	1:40	1:80

* Indicates the dilution of the anti Rh at coating

TABLE 3 Summary of the Results of Bactericidal Tests and Virulence Tests

Strain	Virulence for mice	Growth index*	type 12M protein	Bactericidal index using anti M12 serum	anti-3P 12P and 46P sera
M12-3	low/intermediate	high	present	high	one
M12-13	high	zero	absent	n.d. b	n.d. b
M12-25	high	high	absent	one	high

* The same blood donor used throughout the study
 not done because of low growth index

The titres did not exceed 1:10 in the extracts of original strain, M12/1, M12/2 and M12/3. The titres of the extracts of M12/5-9 varied between 1:20 and 1:80. From M12/11 to M12/25 the titres increased and stabilized between 1:160 and 1:320 (Table 2).

The hemagglutinating capacity of nine of the strains was tested in experiments with red cells coated with KM, 317 or HUN «incomplete» anti-D. The HUN coat useful for detection of the strains.

Bactericidal Effect of Antisera against Strains Repeatedly Passaged in Mice and of Antiserum to Strain M12-3

Strains originally belonging to types M3, M12 and M46 but devoid of type antigens after mouse passages. The effect of anti-3P in indirect bactericidal test is shown in Fig. 1 and in Table 1 similar results were obtained with anti-12P, anti-46P and anti-M1 sera. These sera had no effect in the test with M12/1-3. However, a high bactericidal index was found for the strains M12/14-25.

DISCUSSION

During the mouse passages, several characteristics changed in the type 12 group A streptococcal strain which confirm and extend previous findings (2). Table 3 summarizes the results of some of the experiments. It appears that three major phases represented by strains M12/1-3, M12/4-13 and M12/14-25, during the passages. Clear-

cut differences were observed between strains M12/1-3 as compared to strains M12/14-25, including disappearance of M12 antigen, concomitant increase in mouse virulence and susceptibility in bactericidal test using antisera against heterologous types.

The disappearance of M12 antigen after the fourth passage was observed in the precipitation studies and also in the indirect bactericidal test using anti-type M12 sera. The mouse virulence increased after four passages and remained high during the following passages. In contrast to the increasing

although each strain was tested with blood from the same human donor. Discrepancies between virulence for mice and behaviour in bactericidal tests as found for strains M12/4-13 have also been noted in some group A streptococci by Todd (26) and Lancefield (13).

Conceivably, at least three explanations may be given for the changes in the characteristics of strain 1800 during mouse passages.

1) The strain 1800 might be a mixture of two M-types, e.g. M12 and M1, since anti-M1 serum was bactericidal for strains M12/14-25. Hence, the M1 strain might have been selected because of its higher mouse virulence. This would also be consistent with the finding that M1 has high IgG Fc receptor activity (6). However, this hypothesis implies also that the M3 and M46 strains were contaminated with type M1 since antisera raised against these strains after mouse passages were bactericidal for strains M12/12-25. This objection is valid also for suspected «contamination» of the type 12 strain with any other M1 type. Studies involving clones from single cells of strain 1800 are in progress in order to further exclude the possibility of contamination.

2) The changes in strain 1800 could be caused by

a shift in M type *Maxted & Valkenburg* (18) described an epidemic in which type 12 group A streptococci showed »antigenic drift« towards type 22. However »antigenic drift« occurring during the mouse passages would imply that the M antigens of three types M3, M12 and M46 had changed to one and the same type antigen during the passages i.e. type 1, since anti M1, 3P, 12P and 46P were all bactericidal for strains M12/14-25.

3) Mouse passages of some group A streptococci of defined M type might occasionally lead to selection of variants which lack M antigen but have a hitherto unknown virulence factor in common. *Wittner* (27) using strains which had been serially passed through mice found that purified M proteins had the capacity to absorb opsonic antibody from a variety of heterologous antisera prepared against whole cells or purified M proteins. This absorption also separated passive mouse protecting antibodies and passive hemagglutinating antibodies from opsonic antibodies. The author interpreted these observations as caused by shared determinants among M proteins of different serotypes. However *Fischetti* (7) (who did not use mouse passaged strains) found only limited structural relatedness among three purified M proteins examined and found no evidence of an anti phagocytic activity caused by a peptide sequence common to M proteins. *Fleck* (8) found anti type 6 and anti type 1 serum to protect mice against infection with type 18 streptococci suggesting »an antibody other than M which could protect mice and enhance phagocytosis«.

Several reports have indicated the existence of other virulence factors than the hyaluronic acid capsule and M protein. *Becker et al.* (1) found that mouse passaged strains were rich in M protein and hyaluronic acid and concomitantly virulent for mice. However group A streptococci which had been selected after incubation with human blood

discrepancy with respect to mouse virulence. *Becker et al.* (1) suggested the existence of as yet unidentified streptococcal virulence factors in addition to M protein and capsule. We have found that the M12/25 strain retained the virulence for mice

Any pathogenic significance of the streptococcal receptors for human IgG Fc fragment has not so far been shown. However increase in reactivity for IgG Fc fragment was concomitant with increase in mouse virulence in strains passed on mice. In this

context it is of interest that *Perkins & Hahn* (14) found the phagocytosis of group A streptococci in human blood to be enhanced by antibody derived from rabbit immunoglobulins by pepsin digestion as compared with undigested antibody. *Saito* (22) digested rabbit streptococcal antibody with papain; the digested univalent antibody retained the indirect bactericidal activity *in vitro* and was as effective as intact anti M antibody. Thus the Fc fragment of IgG does not contribute to the phagocytosis of group A streptococci in contrast to the phagocytosis of other bacteria (19).

In conclusion loss of original M antigen concomitant with increasing virulence for mice has been demonstrated in a type 12 group streptococcal strain during mouse passages. Antisera against type M1 and three other heterotypes subjected to mouse passages were found bactericidal for the strains obtained from passages of the original type 12 strain. The nature of the structure responsible for virulence of the passage strain is not known. However the possible role of the streptococcal IgG Fc receptor should be considered.

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a shift in M type *Maxted & Valkenburg* (18) described an epidemic in which type 12 group A streptococci showed «antigenic drift» towards type 22. However, «antigenic drift» occurring during the mouse passages would imply that the M antigens of three types, M13, M12 and M46 had changed to one and the same type antigen during the passages: i.e. type 1, since anti-M1, 3P, 12P and 46P were all bactericidal for strains M12/14-25.

3) Mouse passages of some group A streptococci of defined M type might occasionally lead to selection of variants which lack M-antigen but have a hitherto unknown virulence factor in common. *Wittner* (27) using strains which had been serially passed through mice found that purified M proteins had the capacity to absorb opsonic antibody from a variety of heterologous anisera prepared against whole cells or purified M proteins. This absorption also separated passive mouse protecting antibodies and passive hemagglutinating antibodies from opsonic antibodies. The author interpreted these observations as caused by shared determinants among M proteins of different serotypes. However, *Fischetti* (7) (who did not use mouse passaged strains) found only limited structural relatedness among three purified M proteins examined and found no evidence of an anti-phagocytic activity caused by a peptide sequence common to M proteins. *Fleck* (8) found anti-type 6 and anti-type 1 serum to protect mice against infection with type 18 streptococci, suggesting «an antibody other than M, which could protect mice and enhance phagocytosis».

Several reports have indicated the existence of other virulence factors than the hyaluronic acid capsule and M protein. *Becker et al* (1) found that mouse passaged strains were rich in M protein and hyaluronic acid and concomitantly virulent for mice. However, group A streptococci which had been selected after incubation with human blood were not virulent for mice although their content of M protein and hyaluronic acid was as high as found in mouse passaged strains. On background of the discrepancy with respect to mouse virulence *Becker et al* (1) suggested the existence of as yet unidentified streptococcal virulence factors, in addition to M protein and capsule. We have found that the M12/25 strain retained the virulence for mice also after and during injection in the test animals of hyaluronidase thereby removing the capsule of the streptococci (*Burova & Ryc*, unpublished observation).

Any pathogenic significance of the streptococcal receptors for human IgG Fc fragment has not so far been shown. However, increase in reactivity for IgG Fc fragment was concomitant with increase in mouse virulence in strains passed on mice. In this

context, it is of interest that *Perkins & Hahn* found the phagocytosis of group A streptococci in human blood to be enhanced by antibody derived from rabbit immunoglobulins by digestion as compared with undigested antibody. *Saito* (22) digested rabbit streptococcal antibody with papain, the digested univalent antibody retained the indirect bactericidal activity *in vitro* and was as effective as intact anti-M antibody. Thus the Fc-fragment of IgG does not contribute to the phagocytosis of group A streptococci to the phagocytosis of other bacteria (19).

In conclusion, loss of original M concomitant with increasing virulence for mice has been demonstrated in a type 12 group streptococcal strain during mouse passages. Sera against type M1 and three other heterologous types subjected to mouse passages were not bactericidal for the strains obtained from passage of the original type 12 strain. The nature of the structure responsible for virulence of the passage strain is not known. However, the possible role of the streptococcal IgG Fc receptor should be considered.

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SEROLOGY OF *CAMPYLOBACTER FETUS* SS *JEJUNI* («RELATED» *CAMPYLOBACTERS*)

Demonstration of Strain-specific and Interstrain related Antigens by Immunelectrophoresis and Co-agglutination

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Kosunen, T. U., Danielsson, D. & Kjellander, J. Serology of *Campylobacter fetus* ss *jejuni* («Related» *Campylobacters*). Demonstration of strain specific and interstrain related antigens by immunelectrophoresis and co agglutination. *Acta path. microbiol. scand. Sect. B* 88: 207-218 1980.

Antisera against two strains of *Campylobacter fetus* ss *fetus* (serotype A) two strains of *C. fetus* ss *intestinalis* (serotypes A and B respectively) and eight strains of *C. fetus* ss *jejuni* were used in

immunoassays. The antigenic properties of *C. fetus* ss *fetus* and *C. fetus* ss *intestinalis* were distinctly different from those of the thermophilic *C. fetus* ss *jejuni* strains as shown both by COA and L-RIE. Serotypes A and B of the two former species were also differentiated in COA tests. The *C. fetus* ss *jejuni* organisms gave the strongest reactions with homologous antibodies. In several interstrain cross reactions were seen. By absorption strain specific COA reagents were obtained. Several reactions of identity indicating cross reactive antigens were also seen with L-RIE within the *ss jejuni* group. These results generally agreed with those obtained by COA. With the use of sublethally heated or autoclaved organisms or sonicates heat labile antigens were differentiated from heat stable ones with the use of COA and L-RIE. The apparent antigenic heterogeneity of *Campylobacter* indicates the importance of their serological grouping e.g. for clinical and epidemiological investigations. COA and immunelectrophoresis techniques can be effectively applied for such studies.

Key words: *Campylobacter* antigens, *Campylobacter* serology, co-agglutination, immunelectrophoresis.

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Campylobacter originally classified as *Vibrio* I have proven to be a heterogeneous group of animals. The species according to Smibert (11) *C. fetus* ss *fetus* (originally called *Vibrio fetus*) *C. fetus* ss *intestinalis* both of which are associated with infectious infertility and abortion in cattle and sheep and *C. fetus* ss *jejuni* associated with diarrhea in man and certain animals (4). The former species are the

genital and intestinal tracts of cattle and sheep (12) and of *C. fetus* ss *jejuni* the intestinal tract of various animals including birds (4). The thermophilic properties of this latter species are an important marker to differentiate them from other pathogenic *Campylobacter*s.

The discovery that *C. fetus* ss *jejuni* («related» *Campylobacter*s) is a common cause of enteritis in man (4, 10) has greatly increased the interest in this genus and a need for improved classification has

become evident Serological classifications, which have greatly aided the understanding of the epidemiology, ecology and pathogenesis of infections with organisms as *Salmonellae*, *Shigellae* *Escherichia coli* and *Neisseria meningitidis* have been tried by some authors Based on autoclaving resistant antigens Berg *et al* (2) divided the *Campylobacters* into three main groups by tube agglutination Slide agglutination of live organisms and complement fixation tests of *C fetus* ss *fetus* strains have also been used (3) Strain specific and common antigens as well as non reactive strains were observed Thus, these studies, and those of the immune response of infected humans (4) have demonstrated complexity and heterogeneity

Co agglutination (COA) and immunoelectrophoresis techniques were recently applied for grouping and typing of *Neisseria meningitidis* (6) and *Neisseria gonorrhoeae* (8, 9) In the present study we have used a similar approach for a serological differentiation of *Campylobacter* strains the main emphasis being on strains from human enteritis patients

MATERIALS AND METHODS

Campylobacter Strains

Twelve strains were used in the study two *C fetus* ss *fetus* two *C fetus* ss *intestinalis* («Montana» strains by the courtesy of Dr Firehammer) and eight of *C fetus* ss *jejuni* strains The designations sources and origins of the strains are listed in Table 1

The «Montana» strains grew well at + 37 and 25 °C but not at + 43 °C All the other strains grew at + 43 °C and 37 °C but not at + 25 °C All strains could be cultured on the selective medium described by Skirrow (10) in 5% O₂ atmosphere but not aerobically or anaerobically They were oxidase and catalase positive Gram negative bacteria which under the microscope

fulfilled the morphological criteria laid down by Sneath (11)

Preparation of Antigens

The *Campylobacter* strains were grown on bo blood agar plates in an atmosphere of 5% O₂ 10% C and 85% N₂ for 48 h The *C fetus* ss *fetus* and *C fetus* ss *intestinalis* strains were incubated at + 35 °C, others at + 43 °C

Antigens for immunization The organisms harvested in sterile saline containing 0.5% formalin adjusted to a concentration of approximately 4-5 x 10⁸ organisms ml⁻¹ according to the McFarland scale Equal parts of this suspension and complete Freund's adjuvant were mixed and used for immunizations

Antigens for absorptions and co agglutination tests The organisms were harvested in 0.15 M saline (PBS) centrifuged down at 3000 x g for 20 min, adjusted to a 10% (w/v) concentration After treatment at + 56 °C for 30 min the whole bacteria used for absorptions either as such (L) treated with formalin (F) as described (5) boiled in a water bath (H) (B) or autoclaved at + 120 °C for 2.5 h (A) absorptions the suspensions were centrifuged at 3000 x g for 20 min and adjusted to a concentration of 25 mg ml⁻¹ (wet weight) Formalin treated cells (F) were for absorptions only

For COA tests the 10% suspensions (L, B) were diluted further with PBS to a concentration of 10% (wet weight) (COA tests with live organisms belong like those treated at + 56 °C This treatment killed than 99% of the organisms and was made as a precaution Such organisms were therefore considered to contain both heat labile and heat stable antigens)

Antigens for immunoelectrophoresis The organisms were harvested in PBS centrifuged down at 3000 x g for 20 min suspended in PBS at a concentration of 10 mg ml⁻¹ (wet weight) and disrupted by sonication in a 100 watt MSE apparatus was used The disruption accomplished by sonication at maximal efficiency for 10 to eight periodic intervals of 20 s The sonicates used either as such (for line rocket IE and rocket IV

TABLE 1 *Campylobacter* Strains Used in the Present Study

Species and identification No	Serotype	Source of isolation	Obtained from
<i>C fetus</i> ss <i>fetus</i> 13823	A 1	Bovine fetus	Bozeman Mon
<i>C fetus</i> ss <i>fetus</i> 14840	A subtype 1	Bovine cervicovaginal mucus	Bozeman Mon
<i>C fetus</i> ss <i>intestinalis</i> 13014	A 2	Ovine fetus	Bozeman Mon
<i>C fetus</i> ss <i>intestinalis</i> 14865	B	Bovine fetus	Bozeman Mon
<i>C fetus</i> ss <i>jejuni</i> VTP 67		Pig	Örebro Sweden
<i>C fetus</i> ss <i>jejuni</i> 372		Human feces	Helsinki
<i>C fetus</i> ss <i>jejuni</i> 113		Human feces	Helsinki
<i>C fetus</i> ss <i>jejuni</i> 105		Human feces	Helsinki
<i>C fetus</i> ss <i>jejuni</i> 441		Human feces	Helsinki
<i>C fetus</i> ss <i>jejuni</i> 341		Human feces	Helsinki
<i>C fetus</i> ss <i>jejuni</i> ATCC 29428		Human feces	ATCC
<i>C fetus</i> ss <i>jejuni</i> ST 8		Human feces	Malmö Sweden

or 1 h (for rocket line IE) or autoclaved for 2.5 h (for line IE)

Immunization White adult rabbits were immunized with one of the 12 *Campylobacter* strains. Initially they were injected subcutaneously with organisms mixed with complete Freund's adjuvant, 1.5 ml and 1.0 ml in the hind and fore leg respectively. A series of four more injections was started 4 weeks later (0.25 ml and 2.0 ml 3-4 days apart). The rabbits were bled 7 days after the last injection. Both antisera and immune sera were stored in small aliquots at -40°C . Sodium merthiolate (1:10 000) was added to the serum which was then stored at $+4^{\circ}\text{C}$.

Preparation of reagents One volume of antiserum was mixed with five volumes of whole *Campylobacter* organisms (formalin treated, boiled or autoclaved (see below)). The concentration of the antigen was 250 mg wet weight. This mixture was incubated at $+37^{\circ}\text{C}$ for 3-4 h after which the serum was recovered by centrifugation at $3000 \times g$ for 20 min. Absorbed sera were stored frozen at -40°C and after thawing were used for preparing COA reagents as described below.

Preparation of reagents The production of protein A-staphylococci and the preparation of reagent staphylococci coated with unabsorbed or absorbed *Campylobacter* antibodies as well as control reagents followed the procedure described before (6, 7, 9). The performance of COA tests. One drop of a 1% suspension (w/v) of reagent staphylococci was mixed on a slide with one drop of the 1% *Campylobacter* suspension.

Results were recorded as 1+ and positive ones as 1+ (1) 2+ (moderate) and 3+ (strong and very strong) (20).

Rocket immunoelectrophoresis (LRIE) This was carried out as described by Sandström & Danielsson (8).

The antigen area was about 75 cm² and the reference antigen gel at the cathodic end of the plate 10 cm². Each antigen agarose area with antigen of the intermediate measured 0.25 cm². They were each separated by a 2 mm thick rectangular agarose gel without antigen. Rectangular gels were made in a mould made up of glass plates separated by a U shaped frame 45 x 1 mm, 2 mm thick. 0.3 ml agarose was carefully added at the bottom (45 mm wide) of the mould. After solidification 0.3 ml antigen-containing gel (0.05 ml antigen 100 mg ml⁻¹ and 0.25 ml agarose) was added on the top and this procedure was repeated for the antigen to be tested. In this way sandwiches of 20 *Campylobacter* strains or more could be layered on top of each other. The gel was removed from the mould and 8 mm wide slices were cut perpendicular to the layers in the middle portion of the gel. One such slice 2 x 8 x

100 mm was lifted into place on the 100 x 100 mm glass plate and placed 2 mm from the line antigen gel 10 x 100 mm. This had been previously made by pouring a mixture of 2 ml agarose and 0.3 ml sonicated *Campylobacter* cells against a brass stopper 12 mm from the cathodic end of the glass plate. Contact gels c. 2 and 7 mm wide were poured after which the rest of the glass plate 75 x 100 mm was covered with a mixture of 13 ml agarose and 0.5 ml *Campylobacter* antiserum.

Rocket line immunoelectrophoresis (RLIE) This was carried out essentially as described by Axelsen *et al.* (1) using the micro modification with 5 x 5 cm glass plates. Agarose and barbital buffer were the same as for LRIE. Antigen wells, antigen containing intermediate gel and reference anti-*Campylobacter* antibody gel were arranged as shown in Fig. 3. The areas covered by gel measured approximately 75.5 and 125 cm² respectively (thickness 1.5 mm). The antigen wells were filled with 10 µl of one of the sonicated antigens. Various concentrations of the antigens were tested and 100 mg ml⁻¹ (referred to the wet weight of the organisms) gave optimal results. The intermediate gel contained 20 µl/cm² of antigen preparations as indicated.

Electrophoresis The barbital buffer in the electrophoresis vessels (ionic strength 0.1 pH 8.6) was changed after each run and the electrophoresis apparatus was cooled with tap water during the test runs. Electrophoresis was run for 16 h with a gradient of 1.5 V cm⁻¹. The plates were then pressed between filter papers, washed in saline and water, pressed again and stained with Coomassie blue (1).

RESULTS

Coagglutination

Reagent staphylococci coated with unabsorbed antibodies from individual immune sera against the *Campylobacter* strains were tested against homologous and heterologous cells L, B or A (See Materials and Methods). The results of these tests are given in Tables 2 & 3.

The strongest COA reactions were always seen with homologous organisms. Three of the *C. fetus* ss *fetus* and ss *intestinalis* strains (serotype A according to Berg *et al.* (2)) (Table 1) were very similarly reacting. They differed markedly from *C. fetus* ss *intestinalis* 14365 (serotype B according to Berg *et al.* (2)). These four strains (with lower temperature optimum) were distinctly different from the thermophilic *C. fetus* ss *jejuni* strains and between the groups only a few weak cross reactions were observed.

Among the thermophilic strains a close antigenic relationship was noted between ATCC 29428 and ST 8. Minor cross reactions were obtained with some of the reagents and the heat stable and/or heat labile antigens of other heterologous strains for example between anti 441 and strains VTP 67 372 341 ATCC 29428 and ST 8.

TABLE 2 Co-agglutination Reactions of *C. fetus* ss *fetus*, *C. fetus* ss *intestinalis*, and *C. fetus* ss *jejuni* Organisms in Tests with Reagent *Staphylococcus* Coated with Unadsorbed Anti-*C. fetus* ss *fetus* and Anti-*C. fetus* ss *intestinalis* Antibodies

Reagent staphylococci coated with anti- <i>Campylobacter</i> antibodies	Co-agglutination reactions obtained with <i>Campylobacter</i> organisms														
	<i>C. fetus</i> ■ <i>fetus</i>			<i>C. fetus</i> ss <i>intestinalis</i>						<i>C. fetus</i> ss <i>jejuni</i>					
	13823			14840			13014			14865			105		
	L ^{a)}	B ^{a)}	A ^{a)}	L	B	A	L	B	A	L	B	A	L	B	A
<i>C. fetus</i> ■ <i>fetus</i> 13823	+++	+++	+++	+++	+++	++	+++	+++	++	-	+	+	-	-	-
<i>C. fetus</i> ss <i>fetus</i> 14840	+++	+++	+++	+++	+++	++	+++	+++	++	+	+	+	+	+	-
<i>C. fetus</i> ss <i>intestinalis</i> 13014	+++	+++	+++	+++	+++	++	+++	+++	++	+	+	+	-	-	-
<i>C. fetus</i> ■ <i>intestinalis</i> 14865	-	-	-	-	-	-	-	-	-	+++	+++	++	-	++	-

a) L = Organisms heated at 56 °C

B = Boiled organisms

A = Autoclaved organisms

^{a)} *C. fetus* ss *jejuni* strains 372 113 441 341, ATCC 29428 and ST 8 gave identical results

Antisera against the thermophilic strains were used for absorptions with heated and non heated antigens. The effectiveness of these absorptions was tested by homologous CoA tests. Absorptions with homologous non heated antigens completely removed all reactivity. Homologous absorptions with autoclaved antigens removed reactivity against autoclaved antigens and in some cases also against cold (Table 3 e.g. anti 441 absorbed with 441 A) in this way the reactivity of heat labile antigens could be differentiated from heat stable ones. For some strains absorptions with autoclaved homologous organisms left reactivity against boiling instant antigens (e.g. anti 105 absorbed with 105 A, Table 3). Results after absorption with boiled antigens indicated that heat labile antigens were not responsible.

Unadsorbed antibodies against strains VTP 67 72 113 and 341 had very few cross reactions and no simple heterologous absorptions strain specific antibodies were obtained. Similar heterologous absorptions of antisera against strains 105 441 and 170C 29428 which showed broader cross reactivity made it possible to make specific reagents also against these strains.

immunoelectrophoresis

LRIE as described by Sandsström & Danielsson (8) was used to study the antigenic relationship between the various *Campylobacter* strains. The arrangement of these tests is shown in Figs 1 & 2. The homologous antigen-antibody system consists of the antibody gel at the anode and the line antigen gel at the cathode with a small rectangular block of antigen-containing agarose perpendicular to the line antigen gel. Well under these circumstances form horizontal precipitin lines fusing with the rocket precipitin lines formed where the rectangular antigen-containing agar block is situated. Comparison with antigens from heterologous strains can be

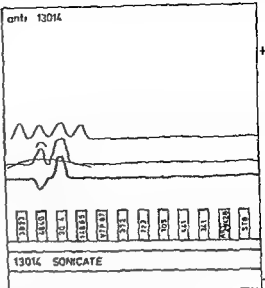


Fig 1 Schematic drawing of a line rocket immunoelectrophoresis with the antigen antibody combination of *C. jejuni* ss *intestinalis* s 13014 with heterologous *Campylobacter* antigens in the intermediate gel. The homologous antigens have formed horizontal precipitin lines fusing with the rockets formed by the homologous intermediate antigen and with rockets of the *C. jejuni* ss *jejuni* and *C. jejuni* ss *intestinalis* group. The thermophilic *C. jejuni* ss *jejuni* strains had no antigens reactive with this antiserum.

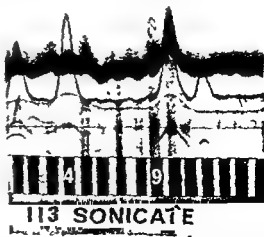
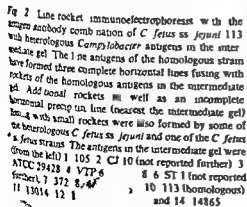


TABLE 2 Co-agglutination Reactions of *C. fetus* ss *fetus*, *C. fetus* ss *intestinalis*, and *C. fetus* ss *jejuni* Organisms in Tests with Reagent Staphylococci Coated with Unadsorbed Anti-*C. fetus* ss *fetus* and Anti-*C. fetus* ss *intestinalis* Antibodies

Reagent staphylococci coated with anti- <i>Campylobacter</i> antibodies	Co-agglutination reactions obtained with <i>Campylobacter</i> organisms													
	<i>C. fetus</i> ss <i>fetus</i>				<i>C. fetus</i> ss <i>intestinalis</i>						<i>C. fetus</i> ss <i>jejuni</i>			
	13823				13014						105			
	L ^a)	B ^a)	A ^b)		L	B	A	L	B	A	L	B	A	VTP 67 ^b)
<i>C. fetus</i> ss <i>fetus</i> 13823	+++	+++	+++		++	+++	++	-	+	+	-	-	-	-
<i>C. fetus</i> ss <i>fetus</i> 14840	+++	+++	+++		+++	+++	++	+	+	+	-	-	-	-
<i>C. fetus</i> ss <i>intestinalis</i> 13014	+++	+++	+++		+++	+++	++	+	+	+	+	+	+	-
<i>C. fetus</i> ss <i>intestinalis</i> 14865	-	-	-		-	-	-	+++	+++	+	-	++	++	-

^a) L = Organisms heated at 56 °C

B = Boiled organisms

A = Autoclaved organisms

^b) *C. fetus* ss *jejuni* strains 372 113 441, 341, ATCC 29428 and ST 8 gave identical results

Antisera of *Campylobacter* strains

105		441			341			ATCC 29428			ST 8		
B	A	L	B	A	L	B	A	L	B	A	L	B	A
		+			+						+		
					+								
+++	+++	+	+	++	+	+		+	+	++	+		
++					+								
+++	+++	+			+			+			+		
++	++												
		++	++	++			++			++	+		+
		+											
		+											
		+	+							+			
					+++	++	++						
					+	++							
+		+++			+++			+++	+++	+++	++	+	+++
		++						+			+		
		++						+					
		+						+++	++	+++	++	+	+++
								+	++		+		
								++	+++	+++	+	+	++
+		++	++		+	+		+	++	++	++	+++	+++

Antisera are given in Figs 1 & 2 and the results of all runs are combined in Table 1

The antigen-antibody combinations for the *C. fetus* ss *fetus* and *C. fetus* ss *intestinalis* strains showed several cross reactions. Strains 13014 and 14865 had specific antigens not shared by the other two. Antisera against these four strains formed

no precipitin lines with antigens from the thermophilic *C. fetus* ss *jejuni* strains

Antisera against the thermophilic strains VTP 67 and 341 gave only one strong precipitin line fusing into the homologous rocket line. In these runs a few cross reactions (CR) were seen with other strains studied but no rocket lines showing reaction of

TABLE 3 Co-agglutination Reactions of Thermophilic Campylobacter Organisms Treated with Heat at 56 ° C. Boiled (B) or Autoclaved (A) Reagent Staphylococci were Sensitized with Unabsorbed or Absorbed Antibodies as Indicated in the Table Reactions Graded as Described in Materials and Methods

Anti Campylobacter antibodies used for coating reagent staphylococci	Absorbing strain	Co agglutination reactions obtained*								
		VTP 67			372			113		
		L	B	A	L	B	A	L	B	A
VTP 67	None	+++	+++	+++		+	+			
	VTP 67-B	b)								
	VTP 67-A		+							
	372-A	+++	+++	+++						
372	None	++			++	++	+++	+		
	372 B									
	ST 8 L				+	++	++			
113	None	+						+++	+++	+
	113-B									
	105-L							+++	+++	+
105	None	+		+	+		+	+		+
	105-B									
	105-A	+								
	441-A	+			+			+		
	VTP 67-Fa)									
441	None			+	+		++			
	441 B									
	441-A									
	341-A									
341	None									
	341-B									
	341 A									
ATCC 29428	None	++			++			++		
	ATCC B				+					
	ATCC B, 441-L									
	VTP 67 L				+					
	VTP 67-L, ST 8-F									
	VTP 67 L, 441-F									
ST 8	None				++	++		+		
	ST 8 B									

a) F = formalin treated organisms

b) Empty space means negative reaction

done by including antigens from these strains in rectangular agar blocks parallelly with the homologous antigen-containing agar block. Cross-reacting antigens will form a rocket fusing with the horizontal precipitin line and rocket line of the homologous system. These cross-reacting antigen-antibody lines are designated CX1, CX2 etc

depending on the number of such lines. Another type of cross-reactions can appear as a rocket line not connected with a horizontal precipitin line probably due to too low a concentration of the antigen in the line gel. Such reactions and cross reactions are designated CR.

A schematic drawing of a photograph of typical

<i>C fetus</i> ss <i>Jejunum</i> ...	CR	CR	CR	CR	CR	CX2	CX1 CX2 CR	CX2 CR	CR	CX2 CR	CX2 CR	CX2 CR
<i>C fetus</i> ss <i>Jejunum</i> 113	CX2					CX2 CX3 CR		CX1 CX2 CX3 CR		CX2 CR		
	CR					CR	CR	CR	CR	CR	CR	CR
<i>fetus</i> ss <i>Jejunum</i> 105						CX2 CX3 CR		CX1 CX2 CX3 CR		CX2 CR		
	CR	CR	CR	CR	CR	CR	CX3 CR	CX2 CX3 CR	CX3 CR	CX2 CX3 CR		CX3 CR
<i>C fetus</i> ss <i>Jejunum</i> 441						CX2 CX3 CR		CX1 CX2 CX3 CR		CX2 CR	CX2 CX3 CR	CX2 CX3 CR
<i>C fetus</i> ss <i>Jejunum</i> 341						CX3 CR		CX3 CR		CX1		
<i>C fetus</i> ss <i>Jejunum</i> ATOC 29428							CX1	CX1	CX1	CX1	CX1 CX2	CX1 CX2
	CR	CR	CR	CR	CR	CR		CR				
<i>C fetus</i> ss <i>Jejunum</i> ST 8	CR	CR	CR	CR	CR		CR	CX1	CR	CX1 CR	CX1 CR	CX1 CR

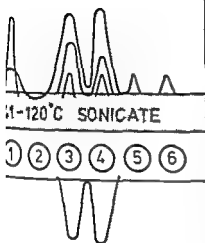
a) See text for abbreviations

b) Empty space means negative reaction

TABLE 4 Results with the Line-racer Immunoelectrophoresis with the Use of Anti-Campylobacter Antisera Tested against Somatized Cells of Homologous and Heterologous Campylobacter Strains in the Intermediate Gel

Antigen in line gel and anti- Campylobacter antiserum	L-RIE reactions with homologous and heterologous Campylobacter antigens in intermediate gel									
	<i>C. fetus</i> ■ <i>fetus</i>		<i>C. fetus</i> ss <i>intest</i>		<i>C. fetus</i> ss <i>jejuni</i>					
	13823	14840	13014	14865	VTP 67	372	113	105	441	341
<i>C. fetus</i> ss <i>fetus</i> 13823	CX1 ^a CX2 CX4	CX1 CX2 CX4	b) CX2 CX4	CX2 CX4						
<i>C. fetus</i> ss <i>fetus</i> 14840	CX2 CX3 CX4 CR	CX1 CX2 CX3 CX4	CX1 CX3 CX4	CX2 CX3 CX4						
<i>C. fetus</i> ■ <i>intest</i> 13014	CX3 CX4	CX2 CX3 CX4	CX1 CX2 CX3 CX4							
<i>C. fetus</i> ■ <i>intest</i> 14865	CX2 CX4	CX2 CX4	CX2 CX4							
<i>C. fetus</i> ■ <i>jejuni</i> VTP 67										

anti ATCC 29428



11-120°C SONICATE

1 2 3 4 5 6

anti ATCC 29428

Heat stable antigens gave precipitation lines. By heating heated or non heated heterologous antigen in the intermediate agar gel we could also show for example that heat labile and heat stable antigens of VP 67 were cross reactive with 105 (Fig 3b). Additional heat labile and heat stable antigens were present in 105 but not in VTP 67. These findings correspond with the results of the COA reactions. Correspondingly R LIE with the anti 441-441 combination with 113 in the intermediate agar gel showed that 441 had at least two precipitating heat labile antigens in common with 372 ATCC 29428 and ST 8 and one heat stable antigen not in common with these strains. With similar R LIE tests we could also prove that 341 contained heat labile antigens reactive with anti ATCC 29428

which is in agreement with COA findings and that ST8 and ATCC 29428 shared heat stable antigens (Fig 3c) which was also found by COA test. In these R LIE tests we also found that ATCC 29428 and ST8 shared an antigen which moved in the cathodic direction (Fig 3c). These findings correspond with the general results of the COA reactions. There were however a few minor discrepancies.

DISCUSSION

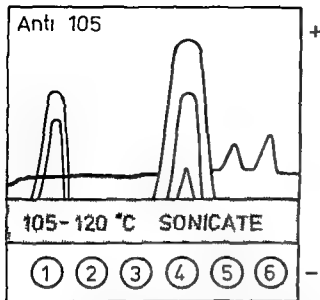
The thermophilic properties of *C. fetus* ss *jejuni* are an important marker to differentiate them from other *Campylobacteria*. However very few markers are available to divide the *jejuni* group further for e.g. clinical epidemiological studies. Berg et al (2) found by tube agglutination tests that this group had an autoclaving resistant common antigen (designated «C») and one out of seven heat labile antigens found in other *Campylobacteria*. Butzler (3) also used agglutination for classification but due to the tendency of these organisms to spontaneously agglutinate or aggregate direct agglutination methods sometimes failed to give dependable results.

Our data show that co-agglutination which is generally not influenced by spontaneous agglutination or aggregation (7) can be applied for serological studies of *Campylobacteria*. By immunizing rabbits with formalin fixed bacteria we obtained antibodies which sensitized Cowan 1 staphylococci to co-agglutinate live or heat inactivated (56 °C) boiled and autoclaved organisms. Prepared reagents distinctly differentiated *C. fetus* ss *jejuni* the thermophilic group from *C. fetus* ss *fetus* and *C. fetus* ss *intestinalis*. The COA tests also differentiated subtype A from the subtype II strains of the *jejuni* *intestinalis* group which is in agreement with the agglutination results reported by Berg et al (2).

Several cross reactions were revealed within the thermophilic group. However heterologous absorptions of the antibodies demonstrated individual differences which indicates heterogeneity within this group. The only pig isolate (VTP 67) was entirely different from the other strains while two strains ATCC 29428 and ST 8 were closely related. The COA method would thus be suitable for assessing identity or non identity i.e. the epidemiological significance of isolates from outbreaks and differences in the distribution of serotypes from different geographical areas.

Line rocket immunoelectrophoresis tests gave further support to the antigenic difference between *C. fetus* ss *fetus* - *C. fetus* ss *intestinalis* group and *C. fetus* ss *jejuni*. This technique as described by Sandstrom & Danielsson (9) allows comparison of

3a



3b

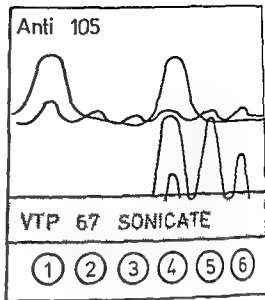


Fig 3a-c Schematic drawings of rocket line immunoelectrophoresis tests with antigen-containing intermediate agar gel. In 3a, the wells 1, 2 & 3 contain sonicated antigens heated at 56 °C boiled and autoclaved, respectively, of strain 441, the wells 4, 5 & 6 correspondingly treated antigens of strain 105. Heat labile antigens of strain 441 have formed 2 rockets. The horizontal line formed by the antigen of strain 105 in the intermediate gel has fused with the rockets formed by boiled and autoclaved antigens of 105 in wells 5 and 6.

In 3b the wells 1, 2 & 3 contain sonicated antigens heated at 56 °C boiled and autoclaved, respectively of VTP 67, the wells 4, 5 & 6 correspondingly treated antigens of strain 105. The antigens of VTP 67 heated at 56 °C in the intermediate agar gel have formed 2 horizontal lines both of which fuse with rocket lines of heat labile antigens of VTP 67 and 105. Additional rockets were formed by heat labile and heat stable antigens of 105.

In 3c the wells 1 & 2 contain sonicated antigens heated at 56 °C and autoclaved respectively of strain 341, the wells 3, 4, 5 & 6 correspondingly treated antigens of strains ST 8 and ATCC 29428 respectively. Strain 341 shared two heat labile antigens with the two latter ones. Note the antigenic similarity of ST 8 and ATCC 29428 both of which also formed a rocket line with a reaction of identity with positively charged heat labile antigens.

identity with the horizontal one. The remaining antisera gave two to three rocket lines fusing with the horizontal lines formed by the homologous antigen. Several reactions of identity were obtained with the other thermophilic strains, including VTP 67 and 341. This would indicate that also VTP 67 and 341 have several antigens cross-reacting with

the other strains, but that their antisera were not optimally reactive to detect these cross reactions in other way. The close relation between the strain ATCC 29428 and ST 8, demonstrated in the CO tests, is also obvious in the IE tests. It should be noted that the homologous antigen antibody combinations of the other thermophilic strains also gave one more precipitin line than the cross reactions of heterologous strains, thus representing strain specific reactions.

Comparison of Results Obtained by Co-Agglutination and Immunoelectrophoresis

By comparing Tables 2, 3 and 4 it can be seen that the COA results generally agreed with those of the L-RIE tests. A few cross reactions were however obtained by the COA tests but not by the L-RIE and vice versa.

Both heat labile and heat stable antigens were responsible for the COA reactions with the homologous antigen antibody combinations. It was also found that cross reactive antigens demonstrated by COA tests could be heat labile or heat stable (Table 2 and 3). Rocket line immunoelectrophoresis (L-RIE) with antigen-containing intermediate agar gel as described by Axelsen *et al.* (1) was therefore used to study the sensitivity to heat of homologous and cross-reactive antigens demonstrated by L-RIE (Table 4). The arrangement of and results with such L-RIE tests are illustrated in Fig 3a-c.

By including unheated or heated (100 or 120 °C) homologous antigens in the intermediate agar gel we could show (Fig 3a) that both heat labile and

ANTIVIRAL AND CELL MULTIPLICATION INHIBITORY ACTIVITIES OF MOUSE INTERFERON PREPARATIONS TESTED ON AN INTERFERON SENSITIVE MURINE SARCOMA CELL LINE

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Abstract. Antiviral and cell multiplication inhibitory activities of mouse interferon preparations tested
on an interferon sensitive murine sarcoma cell line. Acta path microbiol scand Sect. B 88 219-223
1980

The cell multiplication inhibitory effect of SDS treated mouse interferon separated into antiviral (AV)
and cell multiplication inhibitory (CMI) fractions was compared to that of untreated similar interferon
on a line of murine osteosarcoma cells. The untreated interferon preparation and the CMI fractions
independently inhibited the multiplication of the cells as measured by cell count and incorporation of
thymidine into the cultures. The AV fractions containing comparable antiviral activities as the
untreated interferon preparations had only a minor effect on cell multiplication. The biochemical
properties of the fractions studied remain unknown.

Key words: Interferon, murine sarcoma, osteosarcoma, tissue culture.

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Interferon, besides its well-documented antiviral activity, also exerts several biological effects at the cellular level (17). Among these «non antiviral» effects the antitumor effect (13) and the cell multiplication inhibitory effect (7, 12, 16) have been extensively studied. Interferon preparations inhibit the multiplication of both normal and tumor cells *in vitro* (2, 7, 12, 15, 16) and the development of tumors *in vivo* (13) in several experimental systems. Glasgow and co-workers (3, 11) reported that interferon inhibited murine osteosarcomas *in vivo* and *in vitro*.

In most studies the antiviral and the cell multiplication inhibitory activities of interferon preparations have been studied separately. However, separation of these activities has also been reported first by affinity chromatography on an albumin-coupled agarose

column (6) and subsequently by boiling interferon preparations in the presence of sodium-dodecyl sulfate (SDS) followed by gel filtration on a Sephadex G 25 column (4).

In the present study the effects of fractions obtained by the SDS method on murine osteosarcoma cells *in vitro* have been studied.

MATERIALS AND METHODS

Cells

The continuous line of mouse L-929 fibroblast cell originally obtained from the American Type Culture Collection, Rockville, Md. was grown in Eagle's minimal essential medium (MEM) supplemented with 5-10% per cent fetal bovine serum, 300 µg glutamine, 100 µg penicillin and 50 IU streptomycin per ml.

antigens of several strains (20 or more) in one and the same test run. Several cross reactive antigens were demonstrated within the *jejun* group. However, the individual antigenic characteristics demonstrated by COA tests were also found by L-RIE and further documented by R-LIE tests with the antigen-containing intermediate agar gel technique.

COA and IE tests with unheated and heated antigens further documented the antigenic complexity of the *Campylobacteria*. Homologous absorption of the antibodies with autoclaved antigens allowed a differentiation of heat stable antigens from heat labile ones by COA tests. These findings were confirmed by R-LIE tests which also showed that several heat sensitive and heat resistant antigens participated in the reactions. It was also of interest to note that absorptions with boiled bacteria removed reactivity in most cases also against organisms inactivated at 56 °C (Table 2). Whether this is an indication of the presence of capsular (K) antigens in *Campylobacteria* (13) remains to be shown.

The close antigenic relationship between ATCC 29428 and ST 8, demonstrated by COA, was confirmed by immunoelectrophoresis which showed that these strains were the only ones with antigens that moved in the cathodic direction. These findings as well as those with the COA method prove the suitability of these techniques for further serological studies and antigenic analysis of *Campylobacteria*.

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of cells

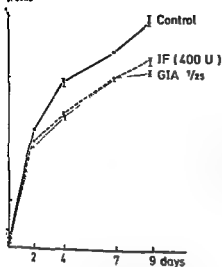


Fig. 2 Growth of osteosarcoma cells in the presence of IF and the CMI fraction. Vertical bars indicate 1 SD.

dissolved in 5 ml Aquasol. The radioactivity in the filterpads was determined with a Mark II liquid scintillation spectrometer (Nuclear Chicago Corp Des Plaines Ill). Cell counts were determined in parallel cultures after trypsin treatment in a haemocytometer.

RESULTS

The SDS treated interferon preparation was fractionated on a Sephadex G-25 column, and eluted with distilled water in 3 ml fractions. Each fraction was tested on L cells for antiviral activity and effect on cell multiplication (Fig. 1). Fractions 6 and 7 contained the major part of AV activity and fractions 13 and 14 the CMI activity. The interferon titre of the AV pool (fractions 6 and 7) was 10000 units per ml and of the CMI pool (fractions 13 and 14) 25 units per ml.

Osteosarcoma cells were cultured in the presence of pooled AV or CMI fractions or interferon preparation. After 3 days' incubation the cell counts were determined. The data presented in Table 1

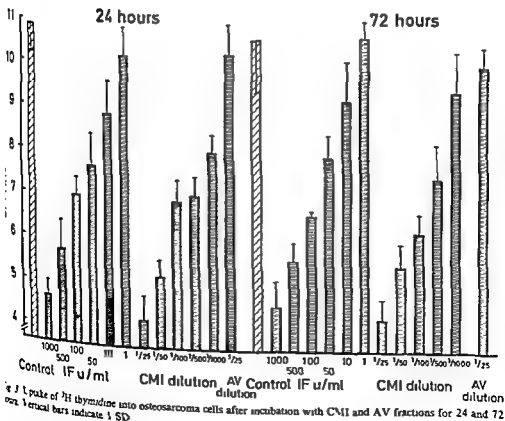


Fig. 3 Uptake of ³H thymidine into osteosarcoma cells after incubation with CMI and AV fractions for 24 and 72 hours. Vertical bars indicate 1 SD.

A mouse osteosarcoma cell line established from a ^{239}Pu irradiation produced primary tumor in C57B1/6 mice (11). The osteosarcoma cells were grown in the same medium as the L cells. In the present experiments the cells were in their 50–70th passage in cell culture. The cells remained tumorigenic when injected into C57B1/6 mice.

Interferon

Murine type I interferon preparation, produced by Dr K Paucker, Medical College of Pennsylvania, Philadelphia, Pa, by exposing L-cells to Newcastle disease virus (NDV), was received from the antiviral program of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. The specific activity of this preparation was about 1×10^7 units per mg protein. Another preparation, produced in L-cells by NDV inoculation, was given to us by Dr G Bodo, E Boehringer Institut für Arzneimittelforschung, Vienna. This preparation contained 1.3×10^6 units of interferon per mg protein. Antiviral activity was measured against Vesicular stomatitis virus (VSV) in L cells by a plaque reduction method (14) or by an infectivity inhibition microtest (5). All titres were compared to international reference standards.

Interferon fractions containing antiviral (AV) and cell multiplication inhibitory (CMI) activities, prepared by SDS treatment, were obtained from H Dahl, Wilhelmsen Institute, University of Oslo. Briefly, interferon preparation was treated with 3.3×10^{-3} M SDS in boiling water bath for 1 minute. After 1 hour at room temperature the preparation was gel filtered on Sephadex G-25 column, and the antiviral and cell multiplication inhibitory activities were tested in each fraction (4, 7). The active fractions were pooled for further studies.

Determination of Cell Proliferation

Osteosarcoma cells were seeded in 24 well plates (Linbro), 5×10^3 cells in each well. Interferon or interferon fractions obtained by SDS treatment were added simultaneously and incubated at 37°C . After various times of incubation the cells were trypsinized, and the number of viable cells was determined after trypan blue staining in a haemocytometer. Each value given in the results is the mean of three or four determinations. Other cultures were fixed with 20 per cent formalin at the end of incubation, washed and stained with methylene blue.

Incorporation of ^3H -thymidine (Tdr) into Cell-cultures

Osteosarcoma cells were seeded in 96 well plates (Linbro), about 5×10^3 cells into each well. Interferon or interferon fractions were added simultaneously. The cells were incubated at 37°C for 24 or 72 hours. At each time period $1 \mu\text{Ci } ^3\text{H-Tdr}$ (40–60 $\mu\text{Ci}/\text{mM}$, New England Nuclear, Boston, Ma) was added to each well and incubated for 4 hours. The supernatant was then discarded, the cells were washed with phosphate buffered saline (PBS), trypsinized and harvested onto filter pads with the aid of an automated cell harvester (Scatron, Flow Laboratories Inc., Ingleswood, Ca). Filterpads were

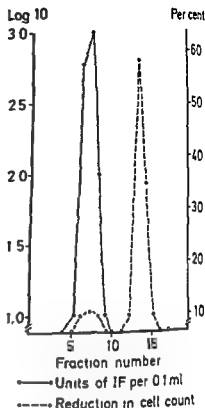


Fig 1 Antiviral (AV) and cell multiplication inhibitory (CMI) activities of fractions obtained from gel filtration on Sephadex G 25 of mouse interferon treated with sodium dodecyl sulfate at 100°C for 1 minute. L cells were grown in the presence of fractions for 3 d. Each fraction was tested at dilution 1:25. ●—● Units of interferon per 0.1 ml. ○—○ Per cent reduction in cell count.

TABLE 1 Effect of Mouse Interferon and the Multiplication Inhibitory and Antiviral Fractions on Multiplication of Osteosarcoma Cells

Preparation, Antiviral activity	Cell count after 3 d of incubation $\times 10^3 \pm$
Control cells	33.3 ± 6.81
Interferon, 400 u/ml	11.6 ± 2.08
Interferon, 10 u/ml	15.6 ± 4.16
AV fraction, 1000 u/ml (1:10 dilution)	23.6 ± 2.10
AV fraction, 400 u/ml (1:25 dil)	28.3 ± 1.53
AV fraction, 10 u/ml (1:100 dil)	29.3 ± 1.58
CMI fraction, 1 u/ml (1:25 dil)	13.0 ± 2.08
CMI fraction, <1 u/ml (1:100 dil)	22.0 ± 0

to these findings. Although the bulk of CMI activity was separated in the low molecular weight fraction, a minor CMI activity was also associated with the AV fractions in most of our experiments. It is possible that the SDS heat treatment selected different molecular species with low CMI activity. On the other hand, the same treatment separated a low molecular weight CMI active component, not necessarily part of the interferon molecule.

Another alternative possibility is that the treatment caused formation of complexes with CMI in SDS alone is cell growth inhibitory. Presence of SDS in the CMI fraction has been excluded (5, 6) but it is possible that the employed method will not detect the hypothetical complexes. Also this hypothesis argues also the finding of highly identical CMI fractions (6) separated from interferon preparation by a different method than presence of SDS.

To solve this question it is mandatory that further separation attempts should be done on pure interferon when sufficient quantities will be available.

Parts of the experimental work have been carried out at the Department of Pediatrics Infectious Diseases Unit, University of Utah Medical Center Salt Lake City, Utah.

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show that interferon and the CMI pool dose dependently inhibited the growth of osteosarcoma cells. The AV pool had only a minor effect on the cell count. Although this effect seems dose dependent the differences were not significant from the controls.

In the next experiments the effect of interferon preparation and the CMI fractions on the growth curve of osteosarcoma cells was examined. As shown on Fig. 2 the suppression by the two agents was comparable. From the fourth day of treatment the number of viable cells was significantly reduced by either agents compared to control cultures.

The growth inhibitory effect could be visually shown when osteosarcoma cells were grown with various concentrations of interferon preparations or AV or CMI fractions and the cultures were stained after 7 days of incubation. Cell growth was clearly suppressed by 50 units of interferon and by comparable dilutions of CMI fractions (1:500). AV fractions had no visible effect on growth.

To further delineate the effect of CMI and AV fractions on osteosarcoma cells the uptake of ^3H TdR into the cells was determined following treatment with serial dilutions for 24 and 72 hours. Serial dilutions of interferon preparation were included for comparison. At the end of the incubation the cultures were pulsed with 1 μCi /well of ^3H TdR for 4 hours. The data in Fig. 3 show that interferon (10000 to 10 units per ml) and CMI fractions (1:25 to 1:1000 dilutions) dose-dependently reduced the uptake of ^3H TdR. AV fractions had only a minor influence on the uptake at 1:25 dilution (containing 400 units of interferon per ml). The degree of inhibition was similar after 24 and 72 hours treatment. The viable counts were not significantly different after 24 hours. After 72 hours the number of cells was dose-dependently reduced by the interferon and by the CMI fractions up to 60 per cent of the controls.

DISCUSSION

The osteosarcoma cells from C57Bl/6 mice were established from a tumor produced by irradiation. Although the presence of C particles has been shown in these cells the significance of these particles is not known. In many respects the murine osteosarcoma seems to be similar to the human osteosarcoma now being treated with interferon with promising results (18). Both systems are highly sensitive to treatment with homologous interferon *in vitro* (11, 19). Therefore this model seems to be well fitted for experimental studies with interferon. The present findings confirm the sensi-

itivity of murine osteosarcoma cells to the growth inhibitory activity of a homologous interferon preparation after up to 70 passages *in vitro*.

A physical separation of antiviral and multiplication inhibitory activities of partially purified human leukocyte and murine fibroblast interferon preparations has been reported from laboratory earlier (4, 6). To confirm these findings we decided to test the active fractions in different cell systems highly sensitive to multiplication inhibitory activity of interferon. Treatment of interferon preparation to separate CMI activity was performed as reported earlier. The CMI and AV activities were demonstrated in the same fractions as previously found in L cells. The low molecular weight fractions containing bulk of CMI activity did not inhibit VSV multiplication in L cells. However, all antiviral fractions contained minor quantities of CMI activity.

In addition to reducing the cell count the fraction containing CMI activity also reduced the incorporation of ^3H TdR into the cells, similarly to the effect of interferon (3). The uptake was not correlated to cell counts. However, after 24 hours treatment with interferon or CMI fractions the cell count was significantly different from that of control cultures. The effect on TdR incorporation is therefore probably not only a function of the cell count but also inhibition of DNA synthesis or possible reduction of the transport of the radioactive compound through the cell membrane (1, 8). Fractions containing AV activity showed only a minor and not significant effect on TdR incorporation.

The interferon preparation employed although partially purified still contained a high proportion of non-interferon substances. One possible explanation for the separation of activities as reported earlier (4, 6) may be that the interferon molecule carries both the AV and the CMI activities and these are separated through the treatment by SDS at 100°C. This hypothesis may not be true since recently it has been reported that apparently homogeneous and pure preparation of murine interferon produced in a similar system as ours still strongly inhibited cell growth (10).

An alternative explanation may be that the CMI activity is a contaminant probably produced together with interferon as a common response. It has been shown (M. Evinger, Nutley, J. personal communication) that the proportions of AV and CMI activities in different molecular species of apparently pure human leukocyte interferon may vary considerably. Some fractions contained low CMI activity together with high AV activity and vice versa. Our data are not contra-

to these findings. Although the bulk of CMI activity was separated in the low molecular weight fractions a minor CMI activity was also associated with the AV fractions in most of our experiments. It is possible that the SDS heat treatment selected for a particular molecular species with low CMI activity while the other hand the same treatment separated a high molecular weight CMI active component, not necessarily part of the interferon molecule. Another alternative possibility is that the treatment caused formation of complexes with CMI and SDS alone is cell growth inhibitory. Presence of the CMI in the low molecular weight fractions.

Thus this hypothesis argues also the finding of seemingly identical CMI fractions (6) separated from interferon preparation by a different method without presence of SDS.

To solve this question it is mandatory that further separation attempts should be done on pure interferon when sufficient quantities will be available.

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EXPERIMENTAL ACUTE PYELONEPHRITIS IN GRIVET MONKEYS, PROVOKED BY *STAPHYLOCOCCUS SAPROPHYTICUS*

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Mårdh P. A., Hovelius B., Melsen F. & Møller B. R. Experimental acute pyelonephritis in grivet
monkeys provoked by *Staphylococcus saprophyticus*. Acta path. microbiol. scand. Sect. B 88: 225-230
1980.

Urinary tract infection by *Staphylococcus saprophyticus* was provoked in two female grivet monkeys. A
non-hemagglutinating strain of *S. saprophyticus* was injected into the renal pelvis of one of the animals
(monkey I) while in the other (monkey II) a hemagglutinating strain of the same species was
inoculated into the bladder by suprapubic puncture. In monkey I massive hematuria and proteinuria
were demonstrated during the post-inoculation (p.i.) week after which the monkey was killed. In
monkey II which was killed after 2 weeks hematuria and proteinuria were present during the first 5

weeks. The kidneys were enlarged and there were signs of acute pyelonephritis. The histopathological examination revealed
microabscesses, interstitial infiltration and numerous leukocytes in the tubules. Both the ureters of
monkey II were congested and microscopically an acute inflammatory reaction was found.
Inflammatory signs were also present in the bladder. Scanning electron microscopy revealed cocci
adhering to the epithelial lining of the urinary tract.

Key words: *Staphylococcus saprophyticus*, experimental pyelonephritis, grivet monkeys.

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Staphylococcus saprophyticus is a coagulase-
negative, novobiocin-resistant species, which is a
common cause of urinary tract infection (UTI),
particularly in young adult women (19, 25).

Most studies on UTI caused by *S. saprophyticus*
have focused on the organism as a causative agent of
acute or chronic hemorrhagic cystitis (11, 21). How-
ever, *S. saprophyticus* often seems to infect the up-
per urinary tract too (5, 10). Patients with this latter
type of infection may complain of flank pain and
have generally palpable kidneys.

neys, yet are often only subfebrile. A reduced
concentration ability in such patients indicates renal
involvement (5, 10).

In rabbits and guinea pigs, Pereira *et al.* (20) was
able to provoke cystitis by instilling *S. saprophyti-*
cus provided a clot formed in the bladder.

In the present study *S. saprophyticus* was
inoculated into the renal pelvis of one female grivet
monkey, while in a similar monkey the organism
was injected into the bladder by percutaneous
suprapubic puncture. Acute pyelonephritis was
demonstrated in both monkeys when studied one

and two weeks post-inoculation (p.i.), respectively. The results of histological, electron microscopic and bacteriological studies are reported

MATERIALS AND METHODS

Animals

Two female grivet monkeys (*Cercopithecus aethiops*), weighing 1.6 kg and 1.8 kg, respectively, were used. The animals, which had been captured in East Africa, were quarantined for 6 weeks, before use.

Organisms

One non-hemagglutinating and one hemagglutinating strain of *S. saprophyticus*, isolated from urine specimens of two women with signs of acute UTI, were used. The strains had been classified as to species according to Kloos & Schleifer (9) and the hemagglutinating ability tested as described elsewhere (4). The hemagglutinating strain had been subcultured once after the primary isolation, while the non-hemagglutinating strain had been subcultured several times and stored frozen, prior to use. The bacteria were incubated overnight in tryptone broth at 37 °C, washed twice in 0.9% saline and resuspended to approximately 10^{10} bacteria/ml.

Experimental Infections

In monkey I, an incision was made through the abdominal wall in the right flank, 2 cm below the costal angle, and the right kidney and renal pelvis were localized. 0.2 ml of the suspension of the non-hemagglutinating strain was injected into the lumen of the right renal pelvis. The abdominal wall was then closed by sutures in three layers and the wound covered with spray-plaster. The operation was performed under aseptic conditions and under general anaesthesia, as previously described (17). Monkey I was re-operated 7 days p.i. The kidneys, the ureters, the bladder and the urethra were extirpated *en bloc* after which the animal was killed.

In monkey II, 0.2 ml of the suspension of the hemagglutinating strain was inoculated directly into the bladder by percutaneous bladder puncture after the suprapubic skin had been disinfected with a 2% iodine tincture. Fourteen days p.i. the same operative procedure was undertaken as in monkey I.

Bacteriological Studies

Before the experimental infection, specimens were collected with a cotton-tipped swab from the back and from the periurethral region of the two monkeys. From each site, two specimens were obtained, one of which was transported in a modified Stuart medium and the other in a selective broth medium containing novobiocin (2 µg/ml) and nalidixic acid (150 µg/ml) (6). The specimens were inoculated onto blood agar plates. All staphylococcal strains isolated were tested for their susceptibility to novobiocin (3). The novobiocin resistant strains were classified according to Kloos & Schleifer (9). In addition, 6 other grivet monkeys were studied in the same way.

Before the animals were infected with *S. saprophyticus*, urine was collected by percutaneous bladder puncture. The procedure was repeated every day until monkeys were killed, viz 1 and 2 weeks respectively. All urine specimens were cultured on blood agar plates. The specimens were also studied for *Mycoplasma* and *Ureaplasma* as described elsewhere (16). Furthermore, tests for proteinuria and hematuria using Neosux® (Ames), and quantitative bacteriological cultures were made after the specimens had been frozen (-20 °C).

At operation (7 days p.i. in monkey I and 14 days in monkey II) specimens were collected from the mucosa of the bladder, the ureter and the renal pelvis by means of cotton-tipped swabs. The specimens were inoculated directly onto blood agar plates.

All staphylococcal strains isolated from the urine, the urinary tract tissue specimens were identified as species, and tested for hemagglutinating ability.

Histological Studies

Specimens from the bladder, the ureters and kidneys, including the renal pelvis, were fixed in 10% formaldehyde and paraffin embedded. Sections 4 µm thick, were cut and stained with hematoxylin and eosin.



Fig. 1. Inflammation of the right kidney of monkey I, extending from the medulla through the cortex ($\times 5$).

Specimens from the bladder, the right ureter and the renal pelvis of monkey II were prepared for scanning electron microscopy (SEM) as described elsewhere (15).

RESULTS

The general condition of monkey I remained unaffected during the experimental period, while monkey II became fatigued and presented slow movements from day 9 p.i. until it was dead on day 14 p.i.

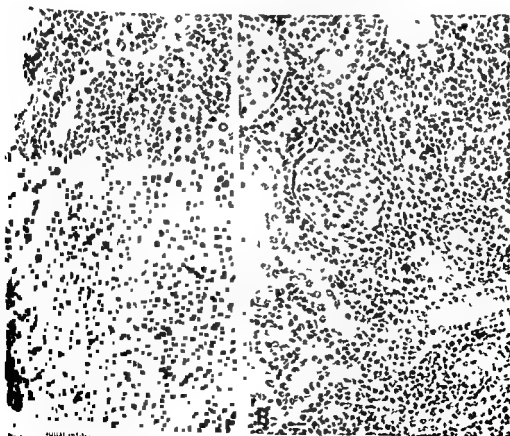
On day 7 p.i., the right kidney of monkey I was found to be moderately enlarged. The right ureter was swollen and congested, while the bladder, the left ureter and kidney were macroscopically normal. In the right ureter and interstitially in the right kidney of monkey I, the histological studies revealed infiltration of polymorphonuclear leukocytes and lymphocytes. Slight cellular infiltration was seen in the bladder epithelium, while the left ureter and the left kidney were normal.

On day 14 p.i., both kidneys of monkey II were swollen and congested. These alterations were more marked in the right than in the left kidney. A small pustule, raised above the surface of the lower pole, was detected at the macroscopic examination of the right kidney (Fig. 1). The cut surface of the kidney revealed patchy areas of suppuration, which were more pronounced in the right kidney. Both the ureters were congested along their entire length, whereas the bladder did not reveal any gross lesions.

Microscopically both kidneys of monkey II showed interstitial infiltration and the tubules were filled with polymorphonuclear leukocytes. In the kidneys, microabscesses were observed, more frequently in the right than in the left organ (Fig. 2A). There was no glomerular destruction (Fig. 2B).

Acute inflammation and cellular infiltration were demonstrated in both the bladder and the ureters (Fig. 3).

SEM preparations of the renal pelvis, the ureter and the bladder showed cocci adhering to the epithelial lining. There was a non randomized distribution in the adherence—that is, cocci (ranging



Renal infiltration with neutrophilic leukocytes and lymphocytes in the tubuli (A) of the right kidney of monkey II. The glomerulus appeared normal (B) ($\times 100$).

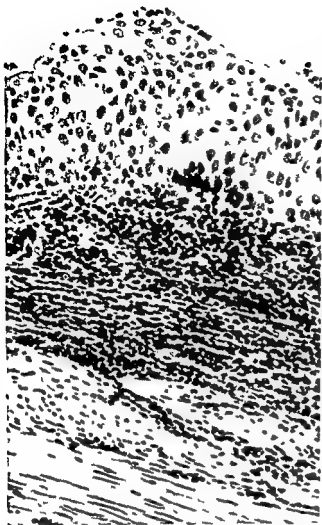


Fig 3 Inflammatory cell reaction mainly with neutrophilic leukocytes and lymphocytes in the bladder wall of monkey II ($\times 100$)

from a few up to 20 bacteria) were attached only to certain epithelial cells

Massive proteinuria and hematuria were found in all urine specimens of monkey I collected after the infection with *S saprophyticus*. In monkey II, slight or moderate proteinuria and hematuria were demonstrated during the first 5 days after the injection of *S saprophyticus* but not in the urine specimens obtained on days 6 through 14 p.i.

perineurethral region of both monkeys, as well as from all the 6 additional animals studied. Half of the 16 specimens transported in the modified Stuart medium and 11 of 16 specimens transported in the selective broth yielded growth of novobiocin-resistant staphylococci. Of the altogether 24 novobiocin-resistant strains isolated, 14 were classified

as *S. cohnii*, 5 as *S. xylosus*, 3 as *S. saprophyticus*, while 2 strains were non-typable. Monkeys I were not among the 3 monkeys from which *S. saprophyticus* was isolated.

The urine specimens collected from the monkeys before the injection of *S. saprophyticus* were sterile. *S. saprophyticus* (10^3 – 10^4 colony forming units (CFU)/ml) was isolated from all urine specimens collected p.i. from monkey I. All strains were non-hemagglutinating and their carbohydrate fermentation pattern was identical with the strain injected.

In monkey II, all 12 urine specimens collected p.i. contained 10^2 – 10^4 CFU/ml of *S. saprophyticus*, which were identical as regards hemagglutinating ability and carbohydrate fermentation pattern with the strain used to infect the animal.

The specimens collected from the bladder and right renal pelvis of monkey I on day 7 p.i. showed growth of *S. saprophyticus*, while no staphylococci were isolated from the left renal pelvis. In monkey II, *S. saprophyticus* was isolated from the bladder, both the ureters, as well as from the left and right renal pelvis in the specimens collected on the day of operation, viz. on day 14 p.i.

In the monkeys, no bacteria except for *S. saprophyticus* were isolated from urine or any of the specimens collected from the bladder and upper urinary tract.

Neither mycoplasmas nor ureaplasmas were isolated from any of the urine specimens obtained from monkeys I and II.

DISCUSSION

Numerous studies on experimental pyelonephritis have been published (7). In the early experiments, rabbits and mice were generally used. In these animals, infection in the kidney is difficult to establish unless obstruction of the urinary flow or alterations of the kidney structure are induced. Infection in unaltered kidneys was established by hematogenous spread of the organism under sterile conditions. In experiments of the latter kind, *Staphylococcus aureus* showed a predilection for the kidney, whereas coagulase-negative staphylococci produced localized lesions in the liver, but not in the kidney (18). Later, rats were the usual experimental animals for studies on pyelonephritis, as a condition could be established in this species with relatively normal urinary tract (7). To our knowledge, monkeys have not been used as experimental animals in studies of staphylococcal infections of the urinary tract.

The present investigation shows that *S. saprophyticus* produced an acute interstitial inflammation

the kidneys in grivet monkeys, in which no urethra had been undertaken to interfere with the urinary flow and in which no lesions in the urinary tract had been induced. The lesions were histologically indistinguishable from acute pyelonephritis in humans. In the kidneys of these monkeys, the usual inflammatory cell infiltration was dominated by polymorphonuclear leukocytes. *S. saprophyticus* also caused inflammatory changes in the bladder and ureters.

In humans an inflammatory reaction in the bladder is a rarely reported complication of UTI. In patients with UTI caused by *S. saprophyticus* sometimes complain of pain, which in the clinical literature suggests a concrement stagnated in the bladder. Whether this pain is due to congestion or inflammatory changes in the ureters of such patients is not known.

It is known about pathogenicity factors of coagulase negative staphylococci. Such factors occurring in *S. aureus* are lacking in *S. saprophyticus*. Factors such as septicemia and meningitis with *S. epidermidis* and other coagulase-negative, novobiocin-sensitive staphylococci, have generally been attributed to occur in compromised hosts. UTI caused by *S. saprophyticus* occurs, however, in healthy women without predisposing disease in the urinary tract (5, 11).

In contrast to other staphylococcal species, including *S. aureus*, *S. saprophyticus* generally does not direct hemagglutination of sheep erythrocytes. The hemagglutinating strain injected into the bladder provoked more advanced pyelonephritic changes than the non hemagglutinating strain, which was injected into the renal pelvis. In the monkeys infected with the former strain, both kidneys showed pathological alterations. In the monkeys infected with the non hemagglutinating strain, only *S. saprophyticus* pathological changes in the kidneys were only found on the injected side. Although the animal infected with the former strain died one week later than that infected with the non hemagglutinating strain, the difference observation might suggest that the hemagglutinin is a virulence factor. In *Proteus mirabilis* a hemagglutinating factor (type IV pili) seems essential for the establishment of experimental pyelonephritis in rats (1).

In humans, *S. saprophyticus* is known to occur as a urinary pathogen only in the urinary tract. In *in vitro* experiments, *S. saprophyticus* adheres in larger numbers to human urothelial cells than do other established urinary tract pathogens (14). This might explain the tropism of *S. saprophyticus* to the urinary tract. When studying urothelial cells from the pig, which is used by *S.*

saprophyticus is unknown, we did not find any selective adhesive propensity in this bacterium, as compared with *S. epidermidis* (2). Urothelial cells from monkeys have not yet been studied in this respect. In the two monkeys studied, however, urinary sediments from both had the same microscopic appearance as those from patients with UTI caused by *S. saprophyticus*, that is, cocci occurred in clusters and were characteristically attached to cellular elements (2, 5).

In spite of signs of acute pyelonephritis, the urine specimens from the monkeys contained only small numbers of bacteria, viz. $< 10^5$ CFU/ml. This finding tallies with studies on women with signs of upper UTI caused by *S. saprophyticus* (5). *S. saprophyticus* has a longer generation time in urine than has *Escherichia coli* (1). Moreover, the cluster-forming tendency of *S. saprophyticus* leads to a discrepancy between the true number of bacteria present in the sample and the actual number of CFU demonstrated on cultures of the specimen. Freezing of the specimens before culture, as done in this study, also gives a reduced number of bacteria as compared with fresh urine specimens.

Coagulase negative, novobiocin-resistant staphylococci occur frequently in the skin flora of a variety of animals. In contrast to humans (8) they seem to predominate there over the novobiocin-sensitive species (13). The skin flora of the grivet monkeys does not seem to differ in this respect from domestic and the more commonly used laboratory animals (13), as indicated by the present study.

In non human primates various species of *Mycoplasma* can be found, including those recovered from the human genital tract (23). *Mycoplasma hominis* has been isolated from the urine of patients with chronic pyelonephritis (12). The possible part played by this species in the etiology of acute pyelonephritis has recently been considered (24). However, neither *M. hominis* nor ureaplasmas were isolated from the two monkeys in our study.

S. saprophyticus injected into the bladder of rats before and after a bladder diverticulum had been prepared did not invariably cause infection (20). In order to ensure that the inoculated bacteria were not rapidly eliminated from the bladder, *S. saprophyticus* was injected together with blood and CaCl_2 in order to produce a clot. When using this technique, no pathological alterations were found in the rats, whereas in guinea pigs and rabbits treated in the same way, vascular dilatation, serous exudation and an inflammatory cell reaction in the bladder wall were demonstrated. *Staphylococcus epidermidis* produced identical results, although less regularly (20).

Clinical studies (5, 10) have indicated that *S. saprophyticus* can cause acute pyelonephritis in

man. However, no histopathological investigation of this condition has yet been reported.

The present study has shown that the grivet monkey is suitable to use as an animal model for the investigation of UTI caused by *S. saprophyticus*. Our study showed that *S. saprophyticus* in such animals provoked infection of both the lower and the upper urinary tract leading to cystitis, ureteritis and pyelonephritis without any lesions in the urinary tract being produced prior to infection.

This study was supported by grant 512 8215 from the Danish Medical Research Council and by grant 16X 4509 from the Swedish Medical Research Council.

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THE ENDOTOXIN-LIBERATING EFFECT OF ANTIBIOTICS ON MENINGOCOCCI *IN VITRO*

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Three strains of *Neisseria meningitidis* (two endotoxin liberating and one *in vitro* variant non liberating) were studied during treatment with MIC and 100 times MIC values of benzylpenicillin and chloramphenicol in a chemically-defined protein free medium. Treatment with the highest dose of benzylpenicillin had the most rapid effect on meningococci, although the antibacterial effect was the same for the two penicillin concentrations after 20 h. Chloramphenicol treatment showed a much slower antibacterial effect. After 2 h of antibacterial treatment an increase of filtrable endotoxin in the medium was found for the endotoxin liberating strains only when the highest penicillin dose was used. During the same period there was a rapid cell death. After 20 hours of treatment, however, the endotoxin liberating strains treated with high and low concentrations of penicillin had a markedly reduced content of filtrable endotoxin compared to the controls and to the cultures treated with chloramphenicol. Antibacterial treatment had no or only minimal effect on the total content of endotoxin in the cultures compared to the untreated controls. The endotoxin non liberating strain had about the same total content of endotoxin as the liberating strains but did not liberate filtrable endotoxin into the medium unless filtered with a much higher pressure through a filter with smaller pore size.

Key words: *Neisseria meningitidis*, free endotoxin, antibiotics.

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The morbidity and mortality associated with infections caused by Gram negative bacteria are in many instances considered to be related to the actions of these bacteria. Fulminant meningococcal septicæmia results in release of potent endotoxin which have been shown to produce diffuse vascular damage in many organs (5).

In vitro studies (2, 3) have demonstrated that meningococci may liberate endotoxin into the medium during growth and that this liberation of endotoxin varies between strains and differs within the serogroup. This difference may perhaps partly explain the various clinical pictures observed in meningococcal diseases.

With increasing incidence of meningococci resistant to sulfonamides, other antibacterial treatments have been used including the penicillins (1). In addition to sulfonamide resistance the meningococci have shown higher virulence during the last ten years, more often causing septicæmia and shock symptoms (1).

The purpose of this investigation is to compare the antibacterial effect of penicillin and chloramphenicol on meningococci grown in a chemically defined protein free medium. We have also investigated the short and long lasting effects of the two antibiotics on endotoxin liberating meningococci compared to a non liberating *in vitro* variant.

man. However, no histopathological investigation of this condition has yet been reported.

The present study has shown that the grivet monkey is suitable to use as an animal model for the investigation of UTI caused by *S. saprophyticus*. Our study showed that *S. saprophyticus* in such animals provoked infection of both the lower and the upper urinary tract leading to cystitis, ureteritis and pyelonephritis without any lesions in the urinary tract being produced prior to infection.

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THE ENDOTOXIN-LIBERATING EFFECT OF ANTIBIOTICS ON MENINGOCOCCI *IN VITRO*

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Andersen, B M & Solberg O The endotoxin liberating effect of antibiotics on meningococci *in vitro* Acta path microbiol scand Sect B 88 231-236 1980

Three strains of *Neisseria meningitidis* (two endotoxin liberating and one *in vitro* variant non liberating) were studied during treatment with MIC and 100 times MIC values of benzylpenicillin and chloramphenicol in a chemically-defined protein free medium. Treatment with the highest dose of benzylpenicillin had the most rapid effect on meningococci although the antibacterial effect was the same for the two penicillin concentrations after 20 h. Chloramphenicol treatment showed a much slower antibacterial effect. After 2 h of antibacterial treatment, an increase of filtrable endotoxin in the medium was found for the endotoxin liberating strains only when the highest penicillin dose was used. During the same period there was a rapid cell death. After 20 hours of treatment, however the endotoxin liberating strains treated with high and low concentrations of penicillin had a markedly reduced content of filtrable endotoxin compared to the controls and to the cultures treated with chloramphenicol. Antibacterial treatment had no or only minimal effect on the total content of endotoxin in the cultures compared to the untreated controls. The endotoxin non liberating strain had about the same total content of endotoxin as the liberating strains but did not liberate filtrable endotoxin into the medium unless filtered with a much higher pressure through a filter with smaller pore size.

Key words: *Neisseria meningitidis*, free endotoxin, antibiotics.

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The morbidity and mortality associated with lesions caused by Gram negative bacteria are in many instances considered to be related to the toxins of these bacteria. Fulminant meningococcal septicæmia results in release of potent endotoxins which have been shown to produce diffuse vascular damage in many organs (5).

In vitro studies (2, 3) have demonstrated that meningococci may liberate endotoxin into the medium during growth and that this liberation of endotoxin varies between strains and differs within a subgroup. This difference may perhaps partly explain the various clinical pictures observed in

With increasing incidence of meningococci resistant to sulfonamides, other antibacterial treatments have been used, including the penicillins (1). In addition to sulfonamide resistance the meningococci have shown higher virulence during the last ten years, more often causing septicæmia and shock symptoms (1).

The purpose of this investigation is to compare the antibacterial effect of penicillin and chloramphenicol on meningococci grown in a chemically-defined protein free medium. We have also investigated the short and long lasting effects of the two antibiotics on endotoxin liberating meningococci compared to a non liberating *in vitro* variant.

MATERIAL AND METHODS

Bacterial Strains

Three strains were studied: two endotoxin-liberating isolates, 270R and 840, and one strain, 840E-, that was a variant from 840 which lost its ability to liberate free, filtrable endotoxin upon subcultivation. The strains were serogroups B and typical meningococci in terms of morphology, growth and acid production from sugar, and they were sulfonamide-resistant.

Medium

The medium was prepared as described previously (2): 9.867 g dehydrated medium 199 with Earle's unmodified salts without sodium bicarbonate, 3 g dextrose, 0.2 g co-carboxylase and 2 g disodium hydrogenphosphate were dissolved in sterile, pyrogen-free water to 1000 ml. The pH was adjusted to 7.5 with 1 M NaOH before filtration through a pyrogen-free prefilter and a membrane filter (2). It was ascertained that all equipment was pyrogen free.

Cell Growth

Precultures were grown for 18 h on heated blood agar at 37 °C in 5 per cent CO₂, washed in sterile pyrogen-free saline (0.9% NaCl) three times, and adjusted to an optical density (OD) of 0.6 at 620 nm equivalent to 10⁸–10⁹ colony-forming units per ml (14).

1.25 ml of each suspension was transferred to 49 ml growth medium. The final cultures were grown at 37 °C in 5 per cent CO₂. After 4 h growth, chloramphenicol (Norsk Medisinaldepot, Oslo, 711201) and benzylpenicillin (A/S Apothekernes Laboratorium, Oslo, lot 9/1979) were added to each of two flasks, giving final concentrations of 0.45 µg/ml and 45 µg/ml chloramphenicol and 0.026 µg/ml and 2.6 µg/ml benzylpenicillin. The lowest concentration of each antibiotic was equal to or close to, the MIC value of each strain investigated; the highest level being approximately 100 times the MIC value (14).

Viable Counts

Viable counts were done by determining the number of colony-forming units (CFU) (2). CFU plotted in the figures were log₁₀ per ml.

Endotoxin Determination

During a growth period of 24 h, aliquots of 5 ml were withdrawn from the culture flasks and filtered with pore sizes 0.45 µm and 0.2 µm respectively (Millipore Millex Disposal filter unit Lot no F 8E063040 and Gelman Acrodisc Lot no 11).

Filtrate and unfiltered samples were stored at -20 °C, or examined immediately.

Limulus lysate test was performed as earlier described.

Negative and positive controls were always included.

Rabbit pyrogen test was carried out according to Pharmacopoea Nordica (11).

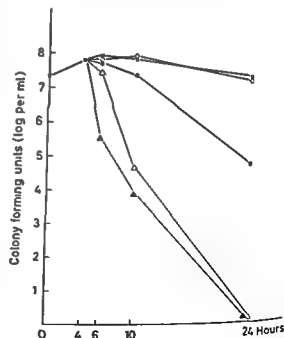
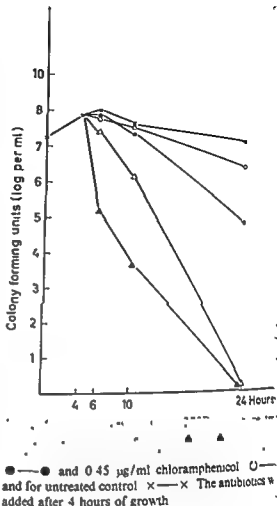


Fig. 2. Growth curves for strain 840 during treatment with 2.6 µg/ml benzylpenicillin ▲—▲, 0.026 µg/ml benzylpenicillin △—△, 45 µg/ml chloramphenicol ●—● and 0.45 µg/ml chloramphenicol O—O and for untreated control x—x. The antibiotics were added after 4 hours of growth.

TABLE 1 Liberation of Meningococcal Endotoxin Through 0.20 μ m Compared to 0.45 μ m Filters

Age of culture hours	Strain		Strain	
	270R		840E-	
	0.20 μ m	0.45 μ m	0.20 μ m	0.45 μ m
0	1:1*	—	—	—
4	1:1000	1:100	1:100	—
6	1:1000	1:100	1:100	—
10	1:1000	1:1000	1:100	—

Endotoxin dilution in the *Limulus* lysate test. — = endotoxin not detectable

RESULTS

Endotoxin Liberation in Medium Containing Antibiotics

The two strains 270R and 840 showed nearly the same 24 h growth curves. The antibiotics had also not the same effect upon the two strains (Fig. 1 and 2). The highest penicillin concentration markedly reduced the viable count already after 2 h, but after 20 h both penicillin concentrations gave similar CFU values. The two chloramphenicol concentrations followed the untreated controls during the first 6 h. Thereafter the highest chloramphenicol concentration significantly reduced the meningococcal CFU.

Effect of Pore Size of the Filters on Endotoxin Liberation

Endotoxin liberation increased when using the smallest filter, 0.2 μ m (Table 1). A relatively high pressure was required to get a filtrate from a cell culture through this filter. The strain 840 E- had more endotoxin in the filtrate from the 0.2 μ m, but not from the 0.45 μ m filter.

When this strain was treated with antibiotics, the highest concentration of penicillin liberated most endotoxin after 2 h when using the 0.2 μ m filter. However, when using the 0.45 μ m filter no endotoxin was found in the filtrates from the cultures of strain 840 E- treated with antibiotics, in contrast to the endotoxin liberating strain 270R.

Endotoxin Liberation During Influence of Antibiotics

In unfiltered samples, the endotoxin titre rapidly increased during the first 6 h of growth. Compared to the controls, antibiotics seemed not to influence the total endotoxin content during the first 10 h of growth (Fig. 3 a & 4 a & 5 a). However, some effect, especially of the penicillins, could be registered after 24 h of growth.

The two strains 270R and 840 showed nearly the

same endotoxin liberation profile and had also the same total content of endotoxin in unfiltered samples during the 24 h period (Fig. 3 a and 4 a). When passed through a 0.45 μ m filter after 2 h of

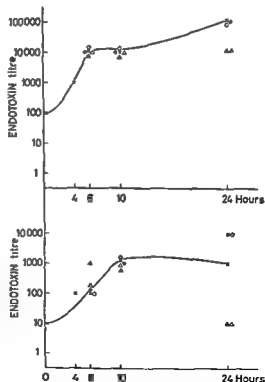


Fig. 3 a Endotoxin in unfiltered cultures for strain 270R during treatment with 2.6 μ g/ml benzylpenicillin \blacktriangle , 0.026 μ g/ml benzylpenicillin \circ , 45 μ g/ml chloramphenicol \triangle , and 0.45 μ g/ml chloramphenicol \bullet , and for untreated control \times .

Fig. 3 b As Fig. 3 a Endotoxin in filtered samples

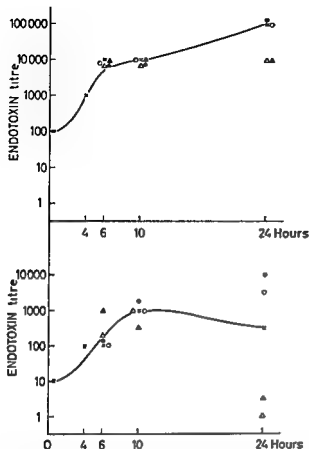


Fig 4 a Endotoxin in unfiltered cultures for strain 840 during treatment with 2.6 µg/ml benzylpenicillin ▲, 0.026 µg/ml benzylpenicillin △, 45 µg/ml chloramphenicol ●, and 0.45 µg/ml chloramphenicol ○, and for untreated control ×

Fig 4 b As Fig 4 a Endotoxin in filtered samples

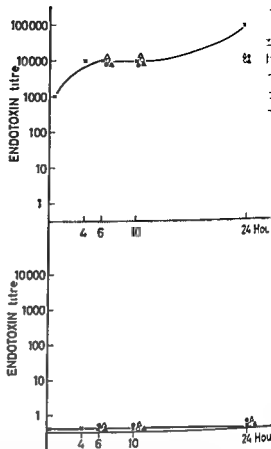


Fig 5 a Endotoxin in unfiltered cultures for strain 840E—during treatment with 2.6 µg/ml benzylpenicillin ▲, 0.026 µg/ml benzylpenicillin △, 45 µg/ml chloramphenicol ●, and 0.45 µg/ml chloramphenicol ○, and for untreated control ×

Fig 5 b As Fig 5 a Endotoxin in filtered samples

antibacterial treatment, the cultures treated with the highest penicillin concentration contained about ten times more filtrable endotoxin than the untreated cultures (Fig 3 b and 4 b). This difference was not

found for the cultures treated with the lowest penicillin concentration, or with chloramphenicol. Four hours later, the content of filtrable endotoxin decreased in the cultures treated with the high

TABLE 2 Endotoxin in Filtrates^a after 2 Hours Antibacterial Treatment of Meningococci: Limulus Test Compared with the Rabbit Pyrogen Test. Strain 270R

Dilution	Control		Penicillin (µg/ml)				Chloramphenicol (µg/ml)			
			0.026		2.6		0.45		45	
	L	R	L	R	L	R	L	R	L	R
1:10	+	-	+	-	+	P	+	-	+	-
1:100	+	-	+	-	+	P	+	-	+	-
1:1000	-	-	-	-	-	-	-	-	-	-
1:10000	-	-	-	-	-	-	-	-	-	-

L = Limulus lysate test, R = rabbit pyrogen test, P = pyrogen response, a) (0.45 µm Millipore) + = endotoxin present, - = no pyrogen response. No entry = Not done

cellular concentration, while the content increased in the other cultures.

After 20 h of treatment the content of filtrable toxin was markedly reduced both for the high and low penicillin concentrations compared to the results from untreated cultures. In comparison the results from the chloramphenicol treated cultures showed even more endotoxin than filtrates from untreated cultures (Fig. 3 b and 4 b). The known non liberating strain 840E- did not liberate endotoxin during treatment with antibiotics but the cultures were passed through a 0.45 μ m filter (Fig. 5 b).

The endotoxin content in filtrates from the toxin-liberating meningococci was tested also in rabbits (Table 2). After antibacterial exposition for 2 h, a pyrogenic response was demonstrated from the highest concentration of penicillin (2.6 μ g/ml) at a 1:100 dilution.

DISCUSSION

Low concentrations were chosen in this investigation, and a rabbit pyrogen test was performed as a control.

It has been shown by others (17) that filters with pore size of 0.22 μ m are suitable for retaining bacteria, but do not hold back endotoxin. In our investigation the filter with the smallest pore size (0.22 μ m) was used.

The cultures were handled more gently when a filter with larger pores was used. As shown earlier (3) using a 0.45 μ m filter corresponded to the results obtained in centrifuged samples (3).

During Influence of Antibiotics
Growth curves showed that treatment with penicillin at 100 times the MIC value had the most rapid effect on growing meningococci.

Endotoxin Liberation During Treatment with Antibiotics

It has been discussed whether the acute bactericidal effect of penicillin at a high concentration may influence the process of endotoxin liberation (16). Too rapid a destruction of Gram negative organisms during therapy was first noted as a possible danger by Reilly *et al* in 1935. Subsequently others have pointed out the possible connection between antibacterial therapy and endotoxin liberation (7, 8). When the meningococci are located mainly in the blood stream high penicillin concentrations may have had a rapid effect on the cell wall. In tissues penicillin will usually not reach the peak serum concentration (4).

The three strains of meningococci studied in this investigation all contained about the same total content of endotoxin in the unfiltered cultures. However when the two endotoxin liberating strains were treated with higher doses of penicillin for 2 h the content of filtrable endotoxin increased. During the same period there was a rapid cell death. This was not found for the other antibacterial treatments having a rather slower effect on the meningococci.

Accordingly when endotoxin liberating meningococci are treated by high doses of penicillin a rapid increase in endotoxin liberation *in vitro* will occur although the total endotoxin content will not change. This effect was not found for the meningococcal strain that did not liberate endotoxin unless it was filtered with high pressure through a 0.2 μ m filter.

If a parallel pattern of endotoxin release upon antibiotic exposure occurs *in vivo* the most severe endotoxin effect may be expected to take place in patients with marked septicaemia when high doses of benzylpenicillin are given intravenously.

Another observation was the gradual reduction of the level of free filtrable endotoxin with time for cultures treated by both penicillin concentrations. After 20 h of treatment free endotoxin was present only at the lowest dilutions whereas the content of endotoxin of the unfiltered samples was rather high for both penicillin concentrations. In contrast the chloramphenicol treated cells seemed to liberate even more endotoxin than the untreated control. The explanation may be that penicillin changes peptidoglycan formation in such a manner that the cells liberate endotoxin and also work as endotoxin adsorbers in the culture. As shown by others endotoxin may adhere to different membranes both *in vitro* and *in vivo* (6, 15) and as earlier shown by us (3) cell wall fragments may form large aggregates of membranes and blebs in the medium.

It has been shown for *Escherichia coli* and *Salmonella typhimurium* (13) that lipopolysaccharide

ride excretion increases markedly when protein synthesis is stopped by addition of chloramphenicol. In our study, the high yield of endotoxin from chloramphenicol-treated meningococci after 20 h of treatment may be explained by a continuing liberation of endotoxin from cells with an intact cell wall that does not work as an absorbant.

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QUANTITATIVE IMMUNOELECTROPHORETIC ANALYSIS OF *SALMONELLA TYPHI* ANTIGENS AND OF CORRESPONDING ANTIBODIES IN HUMAN SERA

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Esperen F, Hertz J B, Høiby N & Mogensen H. Quantitative immunoelectrophoretic analysis of *Salmonella typhi* antigens and of corresponding antibodies in human sera. *Acta path. microbiol. scand. Sect. B* 88: 237-242, 1980.

Quantitative immunoelectrophoretic methods. 86 different antigens were found in sonicated preparations of a *Salmonella typhi* using corresponding rabbit antiserum. Lipopolysaccharide was emulsified and 5 other antigens characterized. Antibodies against a total of 19 *S. typhi* antigens were tested in human sera. The antibody response in patients with typhoid fever was significantly more pronounced compared to that in normal persons. When the antibody response was expressed as a top score, the predictive value of both a positive (precipitin score ≥ 8) test and a negative test as 100% when sera from patients suffering from typhoid fever were compared with sera from normal persons.

Key words: *Salmonella typhi*, crossed immunoelectrophoresis, precipitating antibodies, sero-diagnosis, phage.

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The serology of *Salmonella typhi* and other *Salmonella* has been extensively studied for decades. The main method used is agglutination of whole bacterial cells, but classical immunoprecipitation (Grabar & Williams (8)) and a few other methods have also been applied (30). The accumulated knowledge on the serology of *Salmonella* concerns mainly O, K and H antigens and very useful serotyping schemes have been developed (18, 36). However, cross-reactive bacterial antigens have also been studied (17, 27).

In most laboratories the antibody response to *Salmonella* infections is measured by tests of agglutination of whole bacterial cells (antigen against O, H and Vi antigens, Widal test) but the diagnostic value of such results is sometimes limited.

Later quantitative immunoelectrophoretic methods have been used to investigate the antigenic structure of micro-organisms and to detect precipitating antibodies against a broad spectrum of microbial antigens (2, 10, 11, 15, 22). By these methods a hitherto unknown complex antigenic structure of micro-organisms has been revealed (2, 5, 10, 11, 12, 23, 24, 25). Furthermore, it has been possible simultaneously to investigate the antibody response against a wide range of antigens.

The diagnostic values of the antibody response investigated by these methods has been demonstrated in infections caused by *Pseudomonas aeruginosa* and *Candida albicans* (3, 4, 16).

The purpose of this study therefore is to examine the antigenic structure of *S. typhi* and to measure antibodies in serum from normal persons and patients with typhoid fever.

Antigen preparations *Salmonella typhi* (9 12 - - collection number 58) was kindly provided by Drs I and F Orskov Statens Seruminstitut Copenhagen This strain was selected because it was superior to other *S typhi* strains investigated in inducing human lymphocyte stimulation and it will be used in current studies of the humoral and cellular immune response in *S typhi* carriers

The bacteria were grown 24 hours on solid medium (Truche) and antigens for electrophoretic experiments (Ref Ag) were prepared by sonication as described in detail previously (12) Colloid concentration was 24 g protein/l (refractometry using human IgG as standard)

Commercial lipopolysaccharide (LPS) from *S typhi* was obtained from Difco Laboratories Detroit USA (*S typhosa* 0901 Westphal)

Heat stable antigens were produced by autoclaving the sonicated preparation (1 hour 120 °C) and surface antigens were identified by absorbing the corresponding rabbit antiserum with whole killed (1% formaline in distilled water 37 °C 1 hour) *S typhi* cells as described previously (14)

Rabbit antisera Antiserum (Ref Ab) was produced by immunizing 5 rabbits intracutaneously with 100 µl of the *S typhi* reference antigen in Freund's incomplete adjuvant according to Harboe & Ingild (9) The antiserum was obtained by pooling antisera from successive bleedings after 14 months of immunization The immunoglobulins were isolated and concentrated (9) Before immunization the rabbit sera contained antibodies in low titre against 4 *S typhi* antigens

Rabbit antibodies against common antigen of *Pseudomonas aeruginosa* were prepared as previously described (26)

Human sera 3 groups of human sera were obtained

1) 30 sera from normal persons 15 from blood donors and 15 from persons who had never been immunized with typhoid vaccine or experienced typhoid or paratyphoid fever Median age 27 years (range 3-61 years) 15 male and 15 female

2) 23 sera from 9 patients during typhoid fever Median age 11 years (range 1-33 years) 6 male and 3 female

3) 12 sera from 12 healthy persons who had typhoid fever 2-33 years previously Median age 30 years (range 25-71 years) 3 males and 9 females

Immunoelectrophoretic methods Crossed immunoelectrophoresis crossed immunoelectrophoresis with intermediate gel tandem crossed immunoelectrophoresis and crossed line immunoelectrophoresis were performed on 5 x 5 cm glass plates using Trisbarbital buffer (r/2 = 0.02 pH = 8.6) and 1% agarose from Litex Denmark (Mr = -0.2) (19 20 28)

1 dimension electrophoresis was performed with 1 or 2 µl antigen the intermediate gel contained human sera (20 µl or 40 µl/cm²) bacterial antigen (10 µl or 20 µl/cm²) or saline as a control

Human precipitates were identified by means of the Ag Ab reference pattern and semi quantification was performed by comparing the area of human precipitates

with areas of corresponding rabbit precipitates standard plates (13) containing increasing amounts of rabbit reference antibodies in the intermediate gel 0.5 1 2 5 10 20 and 40 µl/cm² In this way following titres were established 0 < titre 1 ≤ cm² < titre 2 ≤ 0.5 µl/cm² < titre 3 ≤ 1 µl/cm² < titre 4 ≤ 2 µl/cm² < titre 5 ≤ 5 µl/cm² < titre 6 ≤ 10 µl/cm² < titre 7 ≤ 20 µl/cm² < titre 8 ≤ cm² < titre 9

The precipitin scores were calculated according to Axelsen et al (4)

Antigens migrating towards the cathode under given conditions were analysed by means of d immunoelectrophoresis (8)

As protein stain Coomassie brilliant blue was used Staining for lipid and polysaccharide was performed with Oil Red and Periodic Acid Schiff stain respectively (27)

Statistical calculations were carried out using Mann Whitney rank sum test and diagnostic specificity and sensitivity were calculated according to Wulff

RESULTS

86 antigens were reproducibly detected and enumerated in the reference *S typhi* Ag Ab pattern which migrated towards the cathode Antigens against these antigens will not be detected with techniques used In Fig 1 the precipitates of anodically migrating *S typhi* antigens are shown some of these are identified by numbers

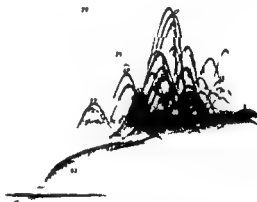


Fig 1 *Salmonella typhi* antigen antibody reference pattern Crossed immunoelectrophoresis with intermediate gel of 2 µl *S typhi* Ref Ag against Ref Ab (20 µl/cm²) Saline in the intermediate gel

84 precipitates are seen 16 precipitates are identified by numbers

(Technical details First dimension electrophoresis anode to the right Second dimension electrophoresis anode at the top Staining Coomassie brilliant blue

TABLE 1 Characteristic Features of some of the 86 *Salmonella* Antigens. Numbers Signify the Antigens in the Reference System

Antigen number	9	47	63	67	77	83
surface						
osmotic		+	+	+	+	+
lipid-positive						+
oil red						+
fast antigen						+
reacts with conom						+
ILPS						+
reacts with conom						+
type of						
protein	+					

Table 1 summarizes the characters of some of the 86 antigens. Antigen no. 83 is heat stable, a surface antigen, PAS-positive, Oil red positive and conom ILPS absorbs antibodies against antigen no.

83 in crossed line immunoelectrophoresis, and therefore it is identified as Lipopolysaccharide

Monospecific rabbit antibodies against 'common antigen of *P. aeruginosa*' precipitated antigen number 9

All human sera contained antibodies against antigen numbers 9, 37, 41, 65 and 83, and antibodies against a total of 19 different *S. typhi* antigens were found in human sera. The numbers of antibody-specificities found in sera from normal persons were in the range 5-7 (median 5), and precipitin scores (Fig. 2) were $3\frac{1}{2}$ -7 (median $4\frac{1}{2}$).

Both the total number of antibody-specificities (median 9, range 7-11) and precipitin scores (median $13\frac{1}{2}$, range 10-17) were significantly higher in sera from patients than in sera from normal persons ($p < 0.01$). Accordingly the predictive value of both a positive test (i.e. patient has typhoid fever) and a negative test (i.e. patient has not typhoid fever) is 100%, if a precipitin score of ≥ 8 -10 is chosen as lower limit of a positive test. No antibody-specificities were solely detected during infection.

Precipitin Score

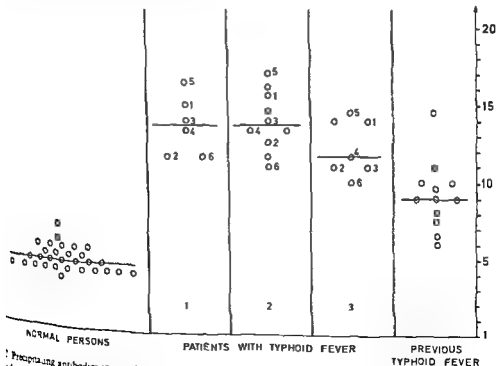


Figure 2. Precipitating antibodies in sera from normal persons and patients with present or previous typhoid fever. The precipitin response is semi-quantified by means of precipitin score paying attention to both the number and titer of precipitates (Archen et al. 1975). 0 = at 0-2 weeks infection, 2 = after 2-4 weeks and 3 = 4-16 weeks. Horizontal line indicates median value. Precipitin scores from 1-4 are indicated by numbers.

was found only in patients with infection b) the total number of precipitins in sera from patients during infections c) Increased precipitin in sera from patients compared to normal sera. d) Infection specific precipitins were found and e) overlapping between normal persons and patients. When total number of precipitins was used as a discriminating method in this material was the precipitin titration taking both number and titres of precipitins into consideration (2). Both the diagnostic sensitivity and specificity of this method was 100%, when it was used to discriminate between normal persons and patients suffering from typhoid fever. The value of the quantitative immunoelectrophoretic methods as an aid during clinical work seems promising. However, whether it is possible to discriminate between various *Salmonella* species and infections with other *Enterobacteriaceae* remains to be investigated.

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CROSS-REACTIONS BETWEEN *SALMONELLA TYPHI* AND 24 OTHER BACTERIAL SPECIES

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Esperen F Hoiby N & Hertz J B Cross reaction between *Salmonella typhi* and 24 other bacterial
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Cross-reactions between antigens from *S. typhi* and 24 other bacterial species were studied by
immunoelectrophoretic methods. A sonicated *S. typhi* antigen preparation and a
corresponding pooled rabbit antiserum regularly presenting 86 immunoprecipitates were used as a
reference system. Antigens from *Enterobacteriaceae* cross reacted intensively with the *S. typhi* antigens.
No cross-reactions between *S. typhi* and antigens from gram positive bacteria were found and only a
few cross-reactions with antigens from gram negative bacteria outside the *Enterobacteriaceae*. The
methodic perspectives of the immunoelectrophoretic approach are stressed by results showing that the
number of cross reacting antigens between *S. typhi* and other *Enterobacteriaceae* was positively
correlated to the number of identical reactions in a collection of 28 biochemical tests.

Key words: *Salmonella typhi*, crossed immunoelectrophoresis, cross reaction, *Enterobacteriaceae*.

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Immunoelectrophoretic methods
have been used to reveal cross reactions between
antigens from a wide spectrum of bacterial species
(1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18). These methods have a high
discriminating power with respect to separation of
antigens and they allow a semi-quantitative expres-
sion of the degree of cross reactions (8).
The antigenic structure of *Salmonella typhi* as
revealed by crossed immunoelectrophoresis has
been described in another article (4) which showed
that 16 different antigens were detectable in the
antigen-antibody pattern.
The purpose of the present study was to identify
Salmonella antigens that are species specific and
antigens that are cross reactive. In addition it was
intended to correlate the number of cross reactive
antigens to the results of biochemical tests normally
used to differentiate *Enterobacteriaceae*.

MATERIAL AND METHODS

Bacteria The *S. typhi* (9 12 - - collection number 58)
reference antigen preparation (Ref. Ag.) and correspon-
ding rabbit antiserum (Ref. Ab.) has been described
elsewhere (4).

Preparation of the different bacterial antigens has been
described previously as well as the origin of most of the
bacteria recorded in Table 1 (5, 8, 11). The *Campylobac-
ter foetus* kindly provided by Dr Mats Walder
Department of Microbiology Malmö General Hospital
Sweden were cultured on solid medium (agar with 5%
horse blood) in micro aerobic atmosphere for 3 days.
Salmonella typhi (9 12 Vi d - collection number 59)
and *Salmonella typhimurium* (1 4 5 12 i 12
collection number 10) were kindly provided by Drs F
and I Orskov Statens Seruminstitut, Copenhagen. *S.
typhi* (9 12 d -) was isolated from a blood culture in
our laboratory. *S. paratyphi A* (FSK 5789/77) and *S.
paratyphi B* (F 3031/77) were kindly provided by Dr K
Gaarslev Statens Seruminstitut, Copenhagen.

TABLE 1 Cross-reactions between *Salmonella typhi* (Ref Ag = 86 antigens) and other Bacterial Species The Number Signify the Antigens in the Reference System The Number of Tested Strains and the Group Type or Collection Numbers Are Given in Parentheses

Cross-reactive antigens and percentage of cross-reactivity				
	100 %	100%—75 %	75%—50 %	50%—25 %
No cross reaction				
<i>Salmonella typhi</i> (1) (9, 12 V, d +)	All other antigens			78
<i>Salmonella typhi</i> (1) (9, 12 d +)	All other antigens			63, 78, 84
<i>Salmonella typhimurium</i> (1) (1, 4, 5, 12, 1, 2)	All other antigens			77, 83, 84
<i>Salmonella paratyphi</i> A (1)	All other antigens			39, 77, 83, 84
<i>Salmonella paratyphi</i> B (1)	All other antigens			56, 77, 83, 84
<i>Escherichia coli</i> (1) (rough)	All other antigens	63		31, 33, 37
<i>Escherichia coli</i> (1) (025 H4)	All other antigens	63		31, 33, 37
<i>Shigella sonnei</i> (1)	All other antigens	77		31, 33, 37
<i>Citrobacter intermedium</i> (1)	All other antigens	45		31, 33, 37
<i>Enterobacter cloacae</i> (1)	All other antigens	50		78, 79, 83, 84
<i>Klebsiella pneumoniae</i> (1) (type 35)	All other antigens			8, 12, 17, 25, 30, 41, 45, 57, 58, 63, 69, 77, 78, 83, 84
<i>Klebsiella ozaenae</i> (1) (195)	All other antigens			8, 12, 17, 31, 33, 39, 41, 57, 58, 77, 78, 83, 84
<i>Proteus mirabilis</i> (1)	All other antigens			8, 12, 17, 27, 29, 33, 41, 77, 78, 83, 84
(17 precipitates not identified)	All other antigens	9		31, 33, 37, 65
<i>Yersinia enterocolitica</i> (1) (type 3)	All other antigens			1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 17, 39, 41, 46, 47, 63, 66, 67, 69, 76, 78, 80, 82, 83, 84
(13 precipitates not identified)	All other antigens	9		1, 2, 3, 4, 5, 6, 7, 8, 12, 17, 39, 40, 41, 63, 69, 77, 78, 83, 84
<i>Serratia marcescens</i> (1)	All other antigens	45		1, 2, 3, 4, 5, 6, 7, 8, 12, 17, 39, 40, 41, 63, 69, 77, 78, 83, 84
(22 precipitates not identified)	All other antigens	9		1, 2, 3, 4, 5, 6, 7, 8, 12, 17, 39, 40, 41, 63, 69, 77, 78, 83, 84
<i>Vibrio cholerae</i> (1) (classica Inaba)	All other antigens			63, 71, 77, 78, 82, 83, 84
<i>Pseudomonas aeruginosa</i> (4) (O groups 3, 5, 6, 11)	1, 75	9		All other antigens
<i>Empylobacter foetus</i> (1)	2, 75			All other antigens
<i>laphy lococcus aureus</i> (1) (E 1369)				All other antigens
<i>Streptococcus pyogenes</i> (1) (group A)				All other antigens
<i>Streptococcus faecalis</i> (1)				All other antigens
<i>Streptococcus pneumoniae</i> (1) (type 19A)				All other antigens
<i>Bacillus cereus</i> var <i>mycoides</i> (1) (ATCC 11778)				All other antigens
<i>Haemophilus influenzae</i> (1) (type b)				All other antigens
<i>Bordetella pertussis</i> (1) (3803, 3825, 3843 and 3860)	10, 71	9		All other antigens
	75, 80			All other antigens

The degree of cross-reactivity between antigens was estimated by comparing the area enclosed of a given precipitate after absorption of antibodies *in situ*, with four immunoplates containing 100, 75, 50 and 25%, respectively, of the original concentration of Ref Ab, as described previously (6, 8, 11). In this way the degree of cross-reactivity of individual antigens with antigens of Ref Ag can be expressed as no cross reaction, 25-50, 50-75, 75-100 and 100% respectively. Cross-reaction to a degree of 25% or less is considered insignificant owing to the analytical variation of the method (9).

The first dimension electrophoresis was run with 2 μ l or 1 μ l of the antigen preparation in question. The intermediate gel of crossed-line immunoelectrophoresis contained 40 μ l/cm² or 20 μ l/cm² of antigen or saline as control. The reference gel contained Ref Ab (20 μ l/cm² or 10 μ l/cm²). Immunoplates compared were always run simultaneously. Technical details of the immunoelectrophoretic experiments are given in another paper (4).

The routine biochemical reaction used in our laboratory to identify *Enterobacteriaceae* to the species level were performed according to *Lautrop et al* (14). 28 tests were included in this study. Nitrate reduction, motility in semi solid medium, fermentation of glucose, mannitol (acid and gas), lactose, rhamnose, arabinose, xylose, maltose, sucrose, adonitol, dulcitol, sorbitol, inositol and salicin, indole production, Voges Proskauer test, growth in the presence of KCN, gluconate oxidation, urease reaction, H₂S production, gelatine (Kohn) liquefaction, decarboxylation of arginine, lysine and ornithine, deamination of phenylalanine, utilization of malonate and utilization of citrate as carbon source.

When using the results of these tests for calculation, we attached the same importance to each test, without considering its value in the diagnosis of the species *Enterobacteriaceae*.

Statistical calculations. The Spearman Rank correlation test was used (significance level = 5%, one tail) (19).

RESULTS

The results of the comparison of antigens from different bacterial species with the *Salmonella* reference system are given in Table 1.

Only 5 antigens proved specific for the *Salmonella* species (no. 8, 12, 41, 77 and 83), and 6 of these were specific for *S. typhi* (no. 77 and 83).

A high degree of cross-reaction between *S. typhi* and the other *Enterobacteriaceae* was found. Most of the antigens cross-reacted 100%. Because of the high number of antigens in the reference pattern, some of the antigens of *Proteus mirabilis*, *Yersinia enterocolitica* and *Serratia marcescens* which precipitated Ref Ab could not be identified. These antigens either cross-reacted less than 100% or not at all. Fig. 1, A-D illustrates the results of cross-reactions with some *Enterobacteriaceae*. The results fit quite well with those of Brown & Brown (1968), who investigated cross-reactions between *Salmonella dublin* antigens and antigens from other *Enterobacteriaceae* (3).

Only a low number of cross-reactions with other negative bacteria outside the *Enterobacteriaceae* was found, and no cross-reactions were found between antigens from *S. typhi* and antigens from other positive bacteria.

The number of identical results of the biochemical reactions performed and the number

TABLE 2. *Immunochemical and Biochemical Comparison of the Reference S. typhi with other Enterobacteriaceae*

	Number of identical biochemical tests (Total 28)	Number of 100% cross reacting antigens (Total 84)
<i>Salmonella typhi</i> (9, 12 V, d -)	27	83
<i>Salmonella typhi</i> (9, 12 d -)	25	80
<i>Salmonella paratyphi</i> II	24	80
<i>Salmonella paratyphi</i> A	22	80
<i>Salmonella typhimurium</i>	22	80
<i>Shigella sonnei</i>	22	72
<i>Escherichia coli</i> (ROUGH)	17	72
<i>Escherichia coli</i> (O25 H4)	20	71
<i>Klebsiella ozaenae</i>	19	71
<i>Klebsiella pneumoniae</i>	14	70
<i>Citrobacter intermedius</i>	19	67
<i>Enterobacter cloacae</i>	14	65
<i>Yersinia enterocolitica</i>	19	
<i>Serratia marcescens</i>	14	
<i>Proteus mirabilis</i>	16	

cross reacting antigens between *S. typhi* and *Enterobacteriaceae* are shown in Table 2. A positive correlation between the number of cross reactions and the number of antigens that react 100% ($r_s = 0.86$, $P < 0.01$)

DISCUSSION

Cross-reaction between *S. typhi* and gram negative bacteria was found. This is in accordance with a previous investigation (18).

S. typhi antigens cross reacted with antigens of *P. aeruginosa*. Høiby found 6 *P. aeruginosa* strains that cross reacted with *S. typhi* (8). 3 of the 6 antigens cross reacted with *Neisseria meningitidis* and Hoff & Høiby found that 4 *N. meningitidis* antigens cross reacted with *S. typhi* (6). Minor differences between the cross reactions with the different antigen antibody reference sera could be due to the different rabbits used.

Antigen found in a wide spectrum of bacterial species designated Common antigen of *Pseudomonas aeruginosa* has been described and characterized previously (20). We have shown (4) that it reacts with antigen number 9 in the *S. typhi* antigen system. This antigen cross reacts with sera from all the gram negative bacteria investigated (Table 1) in accordance with other reports in our laboratory (6, 8, 11, 17).

The close antigenic relationship between *S. typhi* and other *Enterobacteriaceae* was found with more than 40 cross reactive antigens. The results are characterized by the same pattern: either no cross reaction or a 100% cross reaction. This is in accordance with previous investigations (3, 6, 8, 11, 17), which also shows that the closer the structural relationship the higher the number of cross reactions. The apparently species specific antigen number 77 and 83 have both been shown to be identical to lipopolysaccharide stain and antigen number 83 to be identical to Lipopolysaccharide (4). This indicates that there were none of the pure antigens in the sonicated *S. typhi* preparation which was species-specific.

A significant correlation between the number of cross reacting antigens and the number of total biochemical tests was found. This could be a consequence of a correlation between the genotype and phenotypic characteristics: antigenic structure and biochemical capabilities. The relatedness between our reference strain and those bacteria used in this study expressed as number of cross-reacting antigens (Table 1) fits quite well with the relatedness found between *Enterobac-*

teriaceae species by means of DNA reassociation (16).

The high number of 100% cross reactive antigens indicates that many proteins from *Enterobacteriaceae* have a similar structure. Other authors using different serological methods have shown that serological cross reactivity between phylogenetic related proteins is still detectable until 28–40% of the amino acid sequences are substituted. Furthermore the number of amino acid substitutions are correlated to quantitative immunological difference observed when single proteins were studied (15).

Our results show that by using quantitative immunoelectrophoretic methods it is possible to investigate immunological cross reactivity of 86 different antigens. This possibility gives these methods potentials as tools for taxonomic work but the taxonomic implications of the present work should be taken with some reservation until the results have been repeated with a number of international collection strains covering each species.

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BRIEF REPORT

A POSSIBLE ASSOCIATION BETWEEN CFA (II) FIMBRIAE AND K99 FIMBRIAE ON *ESCHERICHIA COLI* AND BACTERIAL ADHERENCE TO HUMAN LYMPHOCYTES

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Feilberg Jørgensen N H & Heron I A possible association between CFA(II) fimbriae and K99 fimbriae on *Escherichia coli* and bacterial adherence to human lymphocytes Acta path microbiol Scand Sect B 88 249-251 1980

We have explored a possible association between *Escherichia coli* binding to human lymphocytes and plasmid coded fimbriae on the bacterial surface. *E. coli* with or without the plasmid coded fimbriae CFA(II) K99 and K88 were mixed with freshly-drawn human peripheral blood lymphocytes. When the lymphocytes were mixed with *E. coli* possessing the CFA(II) fimbriae 59% of the lymphocytes bound bacteria onto the surface whereas only 22% of the lymphocytes bound the CFA(II) derivative. The lymphocytes bound 53% and 56% of two K9⁺ strains whereas 22% and 8% of the lymphocytes bound the same strains without the K99 fimbriae. Twelve per cent and 7% of lymphocytes bound bacteria when the strain was K88⁺ or K88⁻ respectively. Likewise a low (8%) adherence to lymphocytes was found when the *E. coli* did not possess fimbriae or flagella.

Key words: *Escherichia coli* plasmid coded fimbriae human peripheral lymphocytes adherence

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Lymphocytes are divided into two major populations: B lymphocytes and T lymphocytes. The lymphocytes are further subdivided using a number of different methods which depend on differences in the lymphocyte surface structure. It is possible to subdivide lymphocytes by the use of antibodies directed against cell surface antigens, or receptors for the Fc region of IgG, or the ability of binding heterologous erythrocytes. Teodorescu *et al.* (13) have recently described a new method for the subdivision of lymphocytes by which they need the natural binding of eleven different species of bacteria to the lymphocyte surface in order to differentiate different subgroups of lymphocytes. Using this method it has been possible to differentiate between subgroups of human lymphocytes (13) and between subgroups of murine lymphocytes (9). However, since Teodorescu *et al.* workers did not have a structure on the bacterial surface responsible for the adherence of the bacteria to the lymphocyte surface, we thought it worth while to investigate

whether fimbriae may be responsible for adherence of *Escherichia coli* to the surface of human lymphocytes.

Fimbriae are hair like structure which are found on the surface of various gram negative bacteria. Duguid *and co workers* (3) showed as early as 1955 that *E. coli* possessing these structures have the ability to adhere to and agglutinate red blood cells which is today the basis of a test for the bacterial possession of fimbriae. *E. coli* possessing fimbriae may also bind to the surface of other eucaryotic cells (11). Some fimbriated strains of *E. coli* are thus known to be able to adhere to the surface of the epithelium in the small bowel where the adherence of bacteria is considered an important step in the pathogenesis of bacterial intestinal infection. The fimbriae (K88, K99 and CFA(II)) on *E. coli* adhering to intestinal epithelium in pigs (K88) (7), calves (K99) (14) and humans (CFA(II)) (4) differ from other fimbriae by their morphology and biochemical structure and by the observation that the genes for the K88, K99 and CFA(II) fimbriae are localized on plasmids. Furthermore the bacterial agglutination of erythrocytes is mannose

resistant in contrast to the agglutination mediated by the other so called «common fimbriae». It is thus possible to investigate whether there is an association between plasmid coded fimbriae on *E. coli* and bacterial adherence to human lymphocytes, using matched controls (fimbriae negative derivatives of the K88⁺, K99⁺ and CFA(II)⁺ strains). A fimbriae and flagella free strain was also included in the study.

Materials and Methods

Bacterial Strains and Growth Conditions

The strains used in this study were *Escherichia coli* H 10407 (078 K80 H11) is a well-characterized strain possessing the colonization factor antigen (CFA(II)) (4). H 10407-P is a derivative of the strain H 10407 which has lost the ability to produce CFA(II). D 1428 (09 K99⁺) and P 235 (S13) (08 K99⁺ H⁺) are strains possessing the K99 antigen whereas the strains D 1429 (09 K99⁻) and P 235 (08 K99⁻ H⁺) are derivatives of these strains without the K99 antigen. D 751 (0100⁺ K88⁺) and D 753 (0100⁺ K88⁻) are isogenic strains except for the K88 antigen. (1) JE 2217 is a derivative of *E. coli* K12 with no fimbriae or flagella (6).

The bacteria were grown overnight in M 9 broth supplemented with 0.3% vitamin free Casamino acids (Difco) and 0.3% glucose at 37 °C.

The bacteria were harvested by centrifugation, washed twice with PBS containing 0.02% sodium azide (Merck) (PBS-Na₂N₃ buffer) and suspended in the PBS Na₂N₃ buffer at a concentration of about 1×10^7 ml or treated overnight with 5% formaldehyde in PBS, washed with PBS and suspended in PBS at the same concentration.

Determination of Fimbrial Antigens

Slide-agglutination tests were used for the examination of fimbrial antigens (K88, K99 and CFA(II)) before and after the growth of the bacterial cells. CFA(II) antiserum was prepared by immunization of rabbits with living agar grown (M9 agar) H 10407 cells and exhaustive adsorption of the resultant serum with both living and heat killed H 10407 P cells. K88 and K99 antisera were kindly provided by Dr Ida Orskov from the Statens Seruminstitut Copenhagen.

Isolation and Separation of Lymphocytes

Peripheral blood (50 ml) from a healthy volunteer was collected by venous puncture in a screw top bottle containing 10 IU Heparin/ml. The blood was diluted with 0.9% NaCl to 100 ml and the lymphocytes were separated from the remaining part of the blood using Isopaque-Ficoll gradients (Pharmacia). Lymphocytes were washed twice in TC199 with 1% Human serum albumin and thereafter suspended in the same buffer at a concentration of 10^5 /ml.

Assay for the Binding of Bacteria to Lymphocytes

The bacteria and lymphocytes were mixed in a ratio of 200 to 1, the sodium azide concentration was adjusted to 0.02%, and the mixture was centrifuged for 5 min at 500 \times g. The supernatant was decanted, and the lymphocytes were re-suspended in the remaining liquid by vigorously shaking and thereafter examined by phase contrast microscopy.

TABLE 1 Bacterial - Lymphocyte Adherence

Strain	Fimbriae	Percentage of lymph to which <i>E. coli</i> ad.
H 10407	CFA(II) ⁺	59.3 \pm 1.4
H 10407-P	-	22.0 \pm 6.3
D 1428	K99 ⁺	53.7 \pm 10.4
D 1429	-	22.3 \pm 8.9
P 235(S13)	K99 ⁺	56.4 \pm 4.5
P 235	-	7.7 \pm 3.2
D 751	K88 ⁺	12.4 \pm 5.4
D 753	-	7.2 \pm 3.2
JE 2217	-	8.4 \pm 4.1

The results are the mean of at least 4 independent reactions with 3 different donors of lymphocytes.

Results

Lymphocytes isolated from the venous blood of healthy volunteers were assessed for binding different strains of *E. coli*. The results from several assays are summarized in Table 1. Cells binding to more than 6 bacteria were scored as positive. The figure shows examples of adherence between *E. coli* possessing CFA(II) fimbriae and lymphocytes. A lymphocyte without bacteria is also shown.

The adhesion of formalin-treated bacteria to lymphocytes was also explored. No difference was found in results using unfixed or formalin fixed bacteria (data not shown) and results noted in the table are obtained using unfixed bacterial cells. The strain tested for the possession of the fimbrial antigens by slide agglutination after harvest of the bacterial cells. The strains were found to be in possession of the fimbrial antigens.

A mean of 59% of the human peripheral lymphocytes was found to bind bacterial cells to the surface. The *E. coli* strain was CFA(II)⁺. Only 22% of the lymphocytes

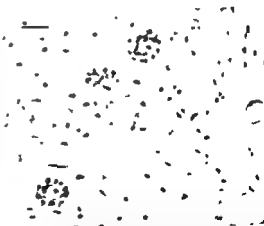


Fig. 1 The figure is a photograph using a phase contrast microscopy showing *Escherichia coli* possessing CFA(II) binding to the lymphocyte surface. One non-binding lymphocyte is also shown. (Scale bar represents 10 μ m).

was found with bacteria bound to the surface when CFA/II strain was mixed with the lymphocytes. 23% of the lymphocytes were bound with K99⁺ bacterial cells adhering to the surface. When the experiments were conducted with strains D 1429 and P 235 (S13) respectively. When strain D 1429 was mixed with the lymphocytes 23% of the lymphocytes bound the bacterial cells. Only 8% of the lymphocytes bound cells from the K99⁺ strain P 235. Applying the strain D 1429 to the human lymphocytes did result in more bacterial cells adhering to the surface than when using the K88⁻ negative strain (1 and 7% respectively). The fimbriae and flagella of strain JE 2217 adhered to about 8% of the lymphocytes.

Adherence of gram negative bacteria to eukaryotic cells. Adherence may be mediated through bacterial surface antigenic structures called fimbriae or pili. Fimbriae are proteins that can be seen in electron micrographs as fine filamentous structures on the bacterial surface (2). Adherence to surface epithelium mediated by fimbriae is considered as an important step in the process of bacterial infections (5, 10, 12). Bacterial adherence may however also be used in the diagnosis and classification of lymphocyte subgroups. Indrani *et al.* (13) have shown that Mayer *et al.* (14) different species of bacteria bind to lymphocytes of human or murine origin. They observed adherence to the lymphocytes from 12% to 40% and were able to classify the lymphocytes into groups. Some lymphocytes bound bacteria from only one species but it was possible to classify lymphocytes on the basis of bacterial binding into subgroups and murine lymphocytes into six groups. Further studies with the same group have shown that classification into these subgroups also correlated with different immunologically functional capabilities.

Bacterial binding to eukaryotic cells may be caused by various fimbrial genes may be chromosomal or located on extrachromosomal elements called plasmids. Bacterial loss of a plasmid results in an accompanying loss of the plasmid-borne characters. An observation that may be used in determining whether a character is plasmid-coded but also whether a certain structure takes part in an observed reaction.

Adherence of enteropathogenic *E. coli*

Adherence of these bound bacterial cells to the surface. Whereas 22% of the lymphocytes adhered to the K99⁺ strain. The difference in adherence observed may be caused by the CFA/II fimbriae on the H 10407 strain or by some unknown character closely connected to the CFA/II fimbriae. Chromosomally coded fimbriae have been found to be responsible for *E. coli* adhering to kidney cells (12) and these fimbriae may be responsible for the adherence of the CFA/II strain to the

lymphocytes, because the strain possesses these fimbriae (5). When the experiments were repeated with the bovine enteropathogenic strains possessing the plasmid-coded K99 fimbriae about 55% of the lymphocytes were found with bacteria adhering to the surface whereas only 22% and 8% of the lymphocytes adhered the K99⁺ strains. The observed binding for the K99⁺ strains to the human lymphocytes could be caused by unknown surface structure but our observations may suggest the existence of a receptor for the K99 fimbriae on the lymphocyte membrane. However there seems to be an association between bacterial possession of CFA/II and K99 fimbriae or some closely related characters and bacterial adherence to human lymphocytes. Furthermore the results with the CFA/II⁺ and the K99⁺ strains imply that there may be overlapping in the lymphocyte subgroups in which the bacterial cells bind.

The fimbria and flagella free strain JE 2217 and the K88⁺ and K88⁻ strains all had a low adherence to the lymphocytes suggesting that these strains did not possess a structure or only minimal amounts of a structure responsible for adherence of these bacteria to lymphocytes.

The results of a closer study of the adherence between the enteropathogenic strains and lymphocytes are in preparation.

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AN ANTIGEN COMMON TO A WIDE RANGE OF BACTERIA

2. A Biochemical Study of a Common Antigen from *Pseudomonas aeruginosa*

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Sompolsky D, Hertz J, B. Høiby N, Jensen K, Manså B, Barkholt Pedersen V & Samra Z. An antigen common to a wide range of bacteria. 2. A biochemical study of a Common Antigen of *Pseudomonas aeruginosa*. Acta path microbiol scand Sect B 88 253-260 1980

Common Antigen (CA) of *Pseudomonas aeruginosa* has been shown to be a protein composed of polypeptide subunits of a molecular weight (MW) of about 62 000. The MW of this protein was estimated to 665 000 by gel filtration on Sepharose CL 6B to 800 000 by electrophoresis on polyacrylamide gradient gels and to about 900 000 by ultracentrifugation on a sucrose gradient. By analytical ultracentrifugation with Schlieren optics a sedimentation coefficient ($S_{20,w}$) of 22.65 was obtained. The isoelectrical point was determined to pH 4.4. The antigen was decomposed on exposure to proteolytic enzymes. Polysaccharide, lipid, deoxyribonucleic acid or ribonucleic acid were not demonstrated in CA. The amino acid content of CA was determined and no hexosamine or abnormal residues were observed. The antigen was degraded when heated to 100 °C for 4 min or when exposed to pH below 4 or above 11 at 4 °C. CA has been isolated from the cytoplasmic water-soluble fraction of disintegrated bacteria and only trace amounts could be obtained from envelope fractions after solubilization with Triton X 100.

Key Words: Common Antigen, *Pseudomonas aeruginosa*, molecular weight, sedimentation analysis, amino acid analysis, enzyme sensitivity.

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Antigens which are shared by a broad spectrum of bacteria which are of interest as immunogens or diagnostic tools (16). One of the macromolecules found in extracts of *Pseudomonas aeruginosa* seems to be the antigen with the broadest spectrum of cross reactions hitherto reported (5, 7, 8, 16). This antigen (Common Antigen or CA) could be obtained in pure form by a combination of salting-out with 18% (w/v) sodium sulfate and gel filtration on Sephadex G 200 (16). In

the present communication some biochemical and biophysical properties of CA are presented.

MATERIALS AND METHODS

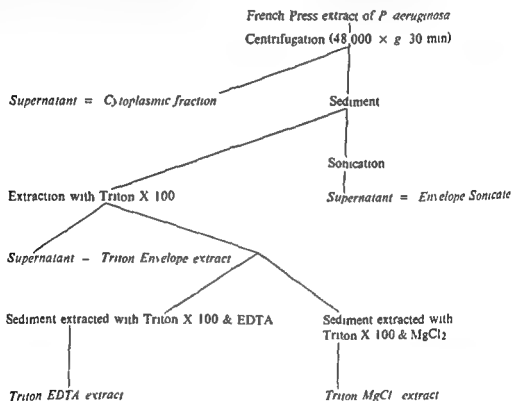
Antigens and Antibodies

Most of the antigens used in the present study (Standard Antigen (St Ag), crude French Press extract and purified Common Antigen) as well as the rabbit immunoglobulins (Standard Antibody (St Ab), anti-CA) have been described previously (16). For this study we

have also prepared antigens from cultures of *P aeruginosa* 888 (16) in fluid Truche medium incubated at 37 °C under constant aeration. Part of the culture was harvested in the exponential growth phase (Log phase Ag) and another part in the late declining phase (Lag phase Ag). We have also examined envelope fractions from bacteria grown on solid Truche medium (9, 16). After disintegration of the bacteria in a French Press (16) remaining whole cells and coars material were removed by centrifugation at $5000 \times g$ for 15 min. Envelopes and membranes were collected by centrifugation at $48000 \times g$ for 30 min. The supernatant was designated cytoplasmic fraction. The pellet was washed three times with 10 mM tris (hydroxymethyl) aminomethane HCl (tris) buffer pH 7.0. A portion of 2.5 g wet weight (ww) was suspended in 2.5 ml dist. H₂O and sonicated ice cooled for three times 45 seconds (20 000 Hz with a Rapidis 300 W equipped with a 9.5 mm tip)

(8, 16). The water soluble part was obtained by centrifugation ($48000 \times g$ for 30 min) and the envelope sonicate. Another portion of 5 g (ww) envelope pellet was suspended in 5 ml 10 mM tris containing 2% Triton X 100 pH 7.2. After sonication overnight at 4 °C the Triton-envelope-extra was centrifuged at $48000 \times g$ for 30 min. The obtained was divided in to two portions both of which were reextracted: one with 2.5 ml 10 mM tris containing 2% Triton X 100 and 1 mM diamine tetraacetate (EDTA) pH 7.2 and the other 2.5 ml 10 mM tris buffer containing 2% Triton and 10 mM MgCl₂ pH 7.2. After incubation at 4 °C the supernatants obtained by centrifugation ($48000 \times g$ 30 min) were designated Triton extract and Triton MgCl₂-extract respectively.

The essential steps in the fractionation procedure are illustrated schematically.



Escherichia coli antigens

Antigens from *E. coli* were prepared by sonication as used for *P. aeruginosa* (16). The bacteria were grown for 48 h at 37 °C on Davis and Mingoli's solidified defined medium (2) either aerobically or strictly anaerobically.

Immunoelectrophoresis with streptococcal antigen was performed as previously described (16).

Amino Acid Analysis

Samples for amino acid analysis as well as for other biochemical investigations were obtained by Na₂SO₄

precipitation and gel filtration on Sephadex G 20 were examined for purity by immunochemical and electrophoresis on SDS polyacrylamide gel. The amino acid composition was determined on a Durrum model D 500 automatic amino acid analyzer. Hydrolysis was carried out 24 h and 77 °C in hydrochloric acid with 0.5% phenol in evacuated ampoules at 110 °C. Norleucine was added as an internal standard. Half cysteine was determined as cysteine after oxidation with performic acid (6) and tryptophan as described by Condon (11).

Polyacrylamide Gel Electrophoresis

The technical details for dodecyl sodium sulphate polyacrylamide electrophoresis (SDS PAGE) were

previously (16). Electrophoresis in gels containing a 2-16% and a 4-30% continuous gradient of urea was used to determine the molecular mass of the native form of CA. The electrophoresis buffer (pH 8.35) contained 0.09 M boric acid, 0.01 M and 0.003 M EDTA (0.003 M). Preelectrophoresis was performed at 70 V for 30 min. Pharmacia sample buffers were used, allowing a maximal sample size of 10 μ l. The application was repeated five times into each well at intervals of 30 min to allow the sample to enter the gel by electrophoresis at 70 V. Thereafter, electrophoresis was continued for 2000 V hours. The gels were either used as first-dimension gels in immunoblots or as second-dimension agarose gels. Gels were stained with 0.7% Naphthol Blue Black (in 7% acetic acid) (60 min at room temp) and then electrophoretically in 7% acetic acid (24 V, 45-60 min). A list of proteins (thyroglobulin 669×10^3 , catalase 440×10^3 , lactate dehydrogenase 140×10^3 and serum albumin 67×10^3) was used to calculate the linear relationship between relative electrophoretic mobility and log of

Isoelectric Focusing of Common Antigen

The isoelectric point (IEP) of Common Antigen was determined by two-dimensional agarose gel electrophoresis of St Ag and purified CA using isoelectric focusing (IEF) in the first dimension and monospecific anti-CA antibody in the second dimension gel. IEF was performed on a Pharmacia model FBE 3000 flat bed electrophoresis apparatus at 8-10 $^{\circ}$ C. Agarose of extremely low electroosmotic flow (HISIF/B) was kindly donated by Latex Glostrup. Cocktails of carrier ampholytes[®] and other technical details were as described for IEF of high molecular weight serum proteins (13). The pH gradient of the IEF gel was determined by suspending pieces of agarose (2 \times 0.5 cm) in 0.5 ml double distilled H₂O. A pH meter with a microelectrode (Blood Micro System 3MK2 Radiometer Copenhagen) was used. Two strips of agarose were cut on both sides of the strip transferred for second dimension electrophoresis. The mean of the pH measurement of corresponding pieces was considered as the actual pH value on the IEF strip.

Extinction Coefficient at 280 nm

Light adsorption at 280 nm of purified CA in 3 mM Tris (pH 7.4) was examined on a Gilford model 2400 S spectrophotometer. Protein concentration was determined by the method of Lowry *et al.* (11) using bovine serum albumin as a standard. The dry weight of CA was determined on lyophilized samples that were desiccated over CaCl₂ at 110 $^{\circ}$ C. After cooling the vessels were weighed on a Mettler analytical balance (\pm 10 μ g) by taking the average of three determinations. One mg protein corresponded to 0.93 mg as measured by dry wt.

Degradation of Common Antigen by Hydrolytic Enzymes

Samples of St Ag and purified CA were incubated with different enzymes and their effect on CA was subsequently analysed by immunochemical methods employing St Ab and anti CA. The enzymes used and the conditions of incubation are given in Table 1. In all cases a control analysis of St Ag or CA incubated with the respective buffers without enzyme was included. The enzymes were dissolved at a high concentration in a concentrated buffer solution so that the substrate never was diluted by more than 10%. After incubation the trypsin digestion was ended by adding apronin (20% v/v). Concerning the other enzymes the activity was either stopped by freezing to -20 $^{\circ}$ C or the immunochemical analyses were carried out immediately after the incubation.

Sensitivity of Common Antigen to Different Hydrogen Ion Concentrations

The crude cytoplasmic extract was dialysed at 4 $^{\circ}$ C against 2000 volumes of buffer solutions of different pH. The stock buffer solution was composed of Citric acid 6.008 g, KH₂PO₄ 3.893 g, boric acid 1.769 g and diethylbarbituric acid 5.266 g in 1 l dist. water (3). This solution was mixed with equal volumes of sodium hydroxide of different concentrations to give the pH values indicated under Results. After dialysis for 24 h

Zonal Centrifugation (17)

100 μ l of 0.5 ml antigen were applied on the top of a sucrose gradient (10-40% w/v in phosphate buffer, pH 7.38). After centrifugation at 23 000 rpm for 1125 min (rotor Spinco SW 50.1) 20 μ l of 0.25 ml were collected from the bottom of the

Flow on Sepharose CL 6B

15 \times 16 cm column (V_L = 180 ml) was used.

was maintained in fractions of human serum containing 150 \times 10³ apoferritin 440 \times 10³ and 669 \times 10³. Fractions of about 1.5 ml collected 6 fractions per hour.

Extinction Coefficient

Extinction analysis was performed with a Beckman Model E analytical ultracentrifuge equipped with optics and with UV optics. An An D rotor at a speed of 6000 rev/min at 24 $^{\circ}$ C was used. The protein was dialysed in 10 mM Tris, 100 mM NaCl at pH 7.4. Extinction coefficient was calculated for water at 25 $^{\circ}$ C.

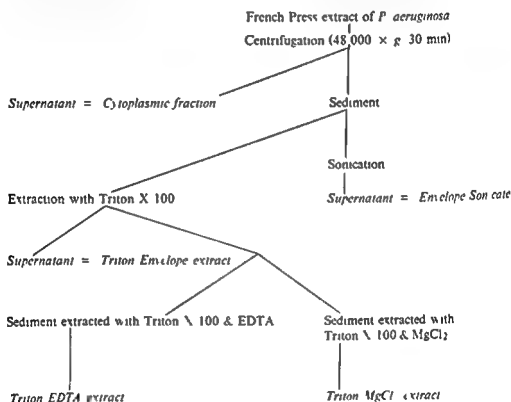
Lectins

Samples of up to 2 mg/ml purified CA were mixed with the mannosyl- and galactophilic lectins of Concanavalin A and examined for inhibition of hemagglutination as described by Gilboa Garber *et al.* (4, 15). Peanut agglutinin and concanavalin A was used by double gel diffusion in agarose (10). As a control we used asialoferritin (peanut agglutinin and concanavalin A) at the same concentration as CA.

have also prepared antigens from cultures of *P. aeruginosa* 888 (16) in fluid Truche medium incubated at 37 °C under constant aeration. Part of the culture was harvested in the exponential growth phase (Log phase Ag) and another part in the late declining phase (Lag phase Ag). We have also examined envelope fractions from bacteria grown on solid Truche medium (9, 16). After disintegration of the bacteria in a French Press (16) remaining whole cells and coars material were removed by centrifugation at 5000 × g for 15 min. Envelopes and membranes were collected by centrifugation at 48000 × g for 30 min. The supernatant was designated cytoplasmic fraction. The pellet was washed three times with 10 mM tris (hydroxymethyl) aminomethane HCl (tris) buffer pH 7.0. A portion of 2.5 g wet weight (ww) was suspended in 2.5 ml dist. H₂O and sonicated ice cooled for three times 45 seconds (20 000 Hz with a Rapidis 300 W equipped with a 9.5 mm tip).

(8, 16) The water soluble part was obtained by centrifugation (48 000 × g for 30 min) and de-enveloped by sonication. Another portion of 5 g (ww) envelope pellet was suspended in 5 ml 10 mM tris containing 2% Triton X 100 pH 7.2. After incubation overnight at 4 °C the Triton envelope-extract was centrifuged at 48 000 × g for 30 min. The supernatant obtained was divided into two portions, both of which were reextracted, one with 2.5 ml 10 mM tris containing 2% Triton X 100 and 1 mM diamine tetraacetate (EDTA) pH 7.2 and the other with 2.5 ml 10 mM tris buffer containing 2% Triton and 10 mM MgCl₂ pH 7.2. After incubation overnight at 4 °C the supernatants obtained by centrifugation (48 000 × g 30 min) were designated Triton extract and Triton MgCl₂-extract respectively.

The essential steps in the fractionation procedure are illustrated schematically.



Escherichia coli antigens

Antigens from *E. coli* were prepared by sonication as used for *P. aeruginosa* (16). The bacteria were grown for 48 h at 37 °C on Davis and Mingoli's solidified defined medium (2) either aerobically or strictly anaerobically.

Immunochemical Methods

Crossed immunoelectrophoresis with intermediate gel (C1w1g) rocket immunoelectrophoresis and fused rocket immunoelectrophoresis with intermediate gel were performed as previously described (16).

Amino Acid Analysis

Samples for amino acid analysis as well as for other biochemical investigations were obtained by Na₂SO₄

precipitation and gel filtration on Sephadex G 200. They were examined for purity by immunochemical criteria and electrophoresis on SDS polyacrylamide gels (1). The amino acid composition was determined with a Durrum model D 500 automatic amino acid analyzer. Hydrolysis was carried out 24 h and 72 h in 5.7 N hydrochloric acid with 0.5% phenol in evacuated ampoules at 110 °C. Norleucine was added as an internal standard. Half-cysteine was determined as cysteine after oxidation with performic acid (6) and tryptophan as described by Condon (11).

Polyacrylamide Gel Electrophoresis

The technical details for dodecyl sodium sulfonate polyacrylamide electrophoresis (SDS PAGE) were given

nosp Ab Monosp Ab

Control CA-Trypsin

Degradation of purified Common Antigen by
For technical details see Table I. Anode to the
the top in first and second dimension electro-
s, respectively

Thermal Sensitivity of CA

Heating of St Ag to 100 °C for 4 min. CA
could not be demonstrated by rocket immunoelec-
trophoresis. Heating to 60 °C for 1.5 or 3 h did not
degrade CA antigenically. Heating isolated
CA at 37 °C for 24 h did not alter the antigenicity
as was observable by C-12 with St Ab or anti-

Stability of CA to Different Hydrogen Ion Concentrations

Exposure of St Ag to a hydrogen ion concentra-
tion of pH 10.03 at 4 °C (see Materials and
Methods) did not seem to have any influence on CA
as could be demonstrated by C-12, but at pH
4.12 it was partially degraded. At pH 4.12 and
pH 10.03 respectively heavy precipitates developed in
dialysis bags, but during dialysis against the
0.02 M phosphate buffer the precipitates
redissolved and electrophoretic mobility and
reactivity seemed unaltered. After dialysis at pH
4.12 CA was immunologically changed as indicated
by the faint wollen immunoprecipitates.

Molecular Weight of CA

On SDS PAGE of purified CA after reduction
with 2-mercaptoethanol at 60 °C for 30 min two bands
were obtained with mobilities corresponding to
MW of 59-62 000 and 62-65 000 Daltons
respectively and both were demonstrable by stain-
ing with Coomassie Brilliant Blue (16). On the
other hand unreduced CA appeared in the void
volume of Sephadex G 200 gel filtration indicating
a MW of > 4-600 000 (16). It was therefore
concluded that the native form of CA was a high
molecular weight complex of one or two subunit
polypeptides. The following experiments confirmed
that the untreated form of CA is a protein of a very
high molecular weight.

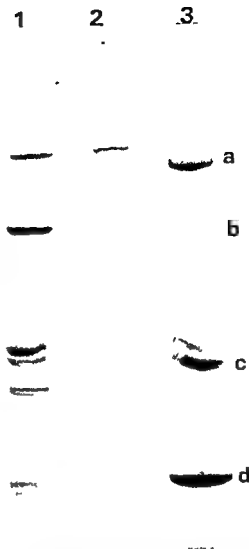


Fig. 2. Polyacrylamide electrophoresis of Common Antigen from *Pseudomonas aeruginosa* on a gradient gel. A 4-30% continuous gradient polyacrylamide gel slab was used. The protein markers (Track 3) were thyroglobulin (MW 669 $\times 10^3$), apoferritin (440 $\times 10^3$), catalase (232 $\times 10^3$) and lactate dehydrogenase (140 $\times 10^3$). Track 2: Purified Common Antigen. Track 1: The supernatant of St Ag after precipitation with 18% Na₂SO₄. Common Antigen was the first antigen to be withheld by the gel.

a. Electrophoresis on 2-16% and 4-30% Continuous Gradient Polyacrylamide gel slabs

The experiments showed that CA migrated a shorter distance than thyroglobulin (MW 669 $\times 10^3$). By extrapolation on the calibration curve of R_f versus log of MW calculated from the bands of the HMW protein kit a MW of 800 000 was estimated.

TABLE 1 The Influence of Hydrolytic Enzymes on Common Antigen Program of Experiments

Enzyme	Activity	Solvent (final conc)	Final enzyme conc after mixture with Standard Antigen or Common Antigen	Incubation Condition
DNase (Bovine pancreas)	1400 Kunitz units/mg protein	45 mM acetate pH 7.0	400 µg/ml	5°C 30 min
RNase (Bovine pancreas)	100 Kunitz units/mg	5 mM tris 1 mM NaCl pH 7.2	200 µg/ml	37°C 2
Muramidase (Lysozyme from egg white Sigma grade I)	17 500-30 000 units/mg	30 mM tris 2 mM EDTA pH 7.5-8.0	17 µg/ml	37°C 2
Trypsin (Bovine pancreas)	Approximately 10 000 BAEE units/mg	10 mM tris pH 7.4	33 µg/ml	35°C 2
Papain	3.5 mAnson units/mg	75 mM Na phosphate 75 mM NaCl 10 mM cysteine 2 mM EDTA pH 7.0	40 µg/ml	35°C 4

the dialysis bags were transferred to 2000 volumes of 0.2M phosphate buffer pH 7.0 and then to the electrophoresis tris barbital buffer of pH 8.6 2 h in each

Temperature Sensitivity of Common Antigen

St Ag and isolated CA were heated to different temperatures and periodically samples were collected and stored in an ice bath till examination

Chemicals

High molecular weight protein kit (HMW) polyacrylamide gradient gels 2/16 and 4/30 and Sepharose CL 6B were obtained from Pharmacia Fine Chemicals AB Uppsala Sweden. All enzymes were products from Sigma Chemical Co. St. Louis Mo. except papain (Merck Darmstadt Germany). Aprotinin (Trasylol 10 000 U/ml) was purchased from Bayer AG Leverkusen Germany. Ampholines[®] were purchased from LKB Produkter AB Bromma Sweden. Peanut agglutinin and soybean agglutinin were donated by Prof. N. Sharon. The Weizmann Institute of Science Rehovot and Pseudomonas lectins by Dr. A. G. Garber Bar Ilan University Ramat Gan Israel.

RESULTS

Growth Phase and Cultivation Conditions

Log phase Ag and Lag phase Ag of *P. aeruginosa* were by Ciwig and rocket immunoelectrophoresis with anti CA found to contain similar concentrations of CA. The same was the case with

antigens prepared from *E. coli* cultures on d medium with no nitrate (2) cultivated under a and strictly anaerobic conditions respectively

The Cellular Localization of Common Antigen

Ciwig of bacterial envelope fractions released considerable amount of CA in the envelope so (see p. 254). However extraction with Triton released huge amounts of the polysaccharide containing antigen related to the O antigen (1) only very little CA. The addition of EDTA MgCl₂ to Triton x 100 had no effect on the release of CA. This is important since some membrane proteins are released by detergent solvents and some proteins from the inner membrane are extracted to a greater extent when EDTA is added to the detergent (12).

Enzymatic Hydrolysis of CA

Samples of St Ag and purified CA were extracted to a number of hydrolytic enzymes and then they were examined for degradation by Ciwig monospecific Ab alongside with control samples kept in the appropriate buffers at the temperature. Deoxyribonuclease, ribonuclease, muramidase had no effect on CA. Trypsin degraded clearly purified CA (Fig. 1) as well as CA in St Ag. Papain had no clear effect on CA in St Ag caused a strong alteration of the electrophoretic mobility of purified CA.

Residues per mol of subunits

Asx acid	44
Asx ^N	27
Asx	27
Asx acid	54
Asx	16
Asx	57
Asx	62
Asx acid	3
Asx	47
Asx ^N	19
Asx ^N	25
Asx	42
Asx	9
Asx	11
Asx	5
Asx	32
Asx	20
Asx acid	4

Assuming that Common Antigen consists of
subunits of a molecular weight of 62 000
The values are the nearest integers of the mean
for 24 h and 72 h hydrolyses except where
indicated
Values for decomposition by extrapolation to zero
hydrolysis
Asx acid as cysteine acid
Asx for 72 h hydrolysis
Asx acid as described by Condon (1)

Extinction Coefficient Isoelectrical Point
Extinction coefficient of CA at 280 nm for a
sol of 10 mg dry wt per ml water E_{1%} was
The isoelectrical point (IEP) measured on St Ag
CA was 4.4 (mean of 4 determinations
pH 4.55)

Amino Acid Composition of Common Antigen
Table 2 indicates the amino acid content of CA
for residues per subunit of MW 62 000
No hexosamine
No residues were observed

DISCUSSION

It is indicated that Common Antigen of
is a protein. It was stained by
Brilliant Blue in polyacrylamide gels. It
is sensitive to the proteolytic enzymes papain and
trypsin.

examined and it reacted with Folin solution of the
Lowry reaction (11). Degradation of the antigenic
structure by a short heating to 100 °C and
sensitivity to very low and very high hydrogen ion
concentrations are also properties compatible with a
protein. The possibility that the molecule is
composed of a protein complexed with a non
proteinaceous substance cannot be excluded but it
should be emphasized that staining for polysaccha
ride and lipid was negative (9). That binding assays
with four lectins gave all negative results that no
hexosamine was observed in the amino acid analysis
chromatogram that DNase and RNase apparently
did not alter its electrophoretic mobility and that
dry wt determination and protein determination by
the method of Lowry *et al* (11) gave compatible
results (see p. 255). CA seems therefore to be a
protein of a MW of 600–900 000 Daltons with a
S₂₀ w of 22.65 an E_{1%} of 10.26 an IEP of pH 4.4
and it is composed of subunit peptides of about
62 000 Daltons.

The molecular weight of CA has been calculated
to somewhat different values depending on the
techniques used. In gradient PAGE it seems clear
that thyroglobulin penetrates to smaller gel pores
than CA. However by gel filtration on Sepharose
Cl 6B CA was eluted later than thyroglobulin. A
possible explanation might be a weak adsorption of
CA to the Sepharose gel. Also the tertiary structure
of CA might be responsible for the discrepancies
noted.

CA was demonstrated in and isolated from the
water soluble fraction that was obtained when
disintegrated bacteria were sedimented at 48 000 ×
g 30 min. The growth phase of the bacteria seemed
of no influence. Extraction of the envelope fraction
with the non ionic detergent Triton X 100 released
only traces of CA and the addition of EDTA or
MgCl₂ did not make this extraction method more
effective. This seems to indicate that no considerable
amount of CA remained attached to the bacterial
membranes. It should however be emphasized that
a substantial yield of CA was obtained by sonication
of the envelope fraction indeed showing that it
contained this substance. The failure of Triton
X 100-containing solutions to extract CA might
indicate that CA in the envelope fraction is present
in the fluid of membrane vesicles and that these are
broken by sonication.

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Education, Denmark to D.S. and from the Danish
Medical Research Council to J.B.H. S. is Established
Investigator of the Head Scientist's Bureau, Ministry of
Health and Efraim Rapoport Professor in Medical
Microbiology, Bar Ilan University, Ramat-Gan, The

St-Ab

Monosp Ab

FRACTIONS FROM SEPHAROSE 6B-CL FIRST FRACTIONS TO THE LEFT

Fig 3 Fused rocket electrophoresis with intermediate gel of fractions from gel filtration on a Sepharose CL 6B column. The sample applied to the column was the supernatant of St Ag after precipitation with 18% Na_2SO_4 . St Ab was added to the rear gel and anti CA to the intermediate gel.

for CA on the 4-30% gel. Electrophoresis of the Na_2SO_4 Ag and of isolated CA did not reveal any proteins of higher MW (Fig 2).

b Gel Filtration on Sepharose CL 6B

By this method CA was also found to be the most high molecular antigen of the crude French Press extract after precipitation with 18% Na_2SO_4 (Fig 3). The calibration curve was calculated for the

relation between elution volume (V_e) and the molecular weight of known proteins. By this method the MW of CA was estimated to 665

c Rate Zonal Centrifugation

Fig 4 shows that CA appeared as the sedimenting substance of Na_2SO_4 Ag. Its ρ in the gradient (fraction 6) was nearly identical of IgM of normal human serum where maximum of α_2 macroglobulin was found in fraction 7.

Sedimentation Coefficient of Common Antigen

From the Schlieren scan a sedimentation coefficient S_{20}^{20} of 22.65 was calculated. Adsorption optics at 280 nm S_{20}^{20} was calculated to 20.8 but a slight portion of the antigen sedimented slowly corresponding to a S_{20}^{20} of 5.45. This phenomenon was obtained repeatedly with samples which were apparently pure. Immunochemical analysis. It is possible that $S_{20}^{20} = 5.45$ corresponds to the CA subunits (16).

Lectin Binding

The possibility that CA is a glycoprotein was examined in a number of tests with known lectins. However, purified CA did not inhibit hemagglutination by two *Pseudomonas* lectins (4, 15) and precipitation was obtained with concanavalin, peanut agglutinin in double diffusion test on agarose (10).

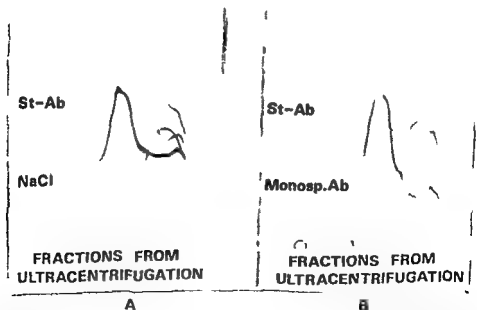


Fig 4 Fused rocket electrophoresis with intermediate gel of fractions of Na_2SO_4 Ag obtained by rate zonal centrifugation (10-40% w/v sucrose). The fractions were applied in the same sequence as collected, i.e. the highest density to the left. Common Antigen seems to be the antigen with the highest density.

BIOTYPES OF CAPSULATED AND NON-CAPSULATED *HAEMOPHILUS INFLUENZAE*

Correlation between Biotypes and β -Lactamase Production

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The C biotypes of capsulated and non-capsulated *Haemophilus influenzae* Correlation between
and β -lactamase production Acta pathol microbiol scand Sect B 88 261-264 1980

Haemophilus influenzae strains were biotyped according to Kilian 393 of the strains were assigned
to I or II while 3 strains remained unclassified Eighty nine per cent of the capsulated strains
had both urease and ornithine decarboxylase biotypes I or IV while 95 per cent of the non-
capsulated strains produced only one of the enzymes biotypes II III or V Of consecutive strains from
upper respiratory tract the incidence of β -lactamase positive strains was higher among capsulated
capsulated β lactamase positive
15 out of 147 non-capsulated
1 of biotype IV and 3 of 7

Words *Haemophilus influenzae* biotypes β lactamase

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basis of three biochemical characteristics
urease activity and decarboxylase
activity K1 an recognized five biotypes of
H. influenzae Most capsulated strains
in biotypes I and II whereas most non-
capsulated strains of biotypes II III and V
of strains from *H. influenzae* infections are in-
duced by capsulated strains and 85-
strains isolated from the upper respiratory
tract are capsulated a rough correlation can be
drawn between biotype and source of infection
(1981)

Present investigation 396 *H. influenzae*
isolated from blood and cerebrospinal fluid
from the upper respiratory tract were biotyped
according to biotype and capsular
status confirmed Furthermore a correla-
tion between β -lactamase production and

MATERIAL AND METHODS

Bacterial Strains

All strains were isolated from routine specimens sent
to our laboratory The material included β lactamase
producing strains consecutively isolated since 1975
consecutive strains isolated from blood and cerebrospinal
fluid since 1976 and consecutive isolates from the upper
tract mainly the nasopharynx Only one strain from
each patient was included Strains from known close
contacts were excluded The strains were preserved at
80 °C in foetal calf serum (Flow Laboratories) with
50% Tryptone Soya Broth (Oxoid) and 1% yeast extract
(Difco)

Biochemical and Serological Tests

The requirement for haemin and nicotinamide adenine
dinucleotide (NAD) was determined by X and V factor
discs on agar plates prepared from 20 g Nutrient Broth
No 2 (Oxoid) and 7 g Bacto-Agar in 800 ml distilled
water Haemolysis was tested on horse blood agar where
a streak of *S. aureus* served as source of V factor (Indole)

authors express their gratitude to Mrs *Anni Bethien* Statens Seruminstitut *Hvidovre Hospital Denmark* Dr *Zvi Kahana* The Weizmann Institute of Science Rehovoth *Uri Kuro* Bar Ilan University and *Simon Waiszbium* Tel Aviv University Israel

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TABLE 3 Distribution of *B* types among Capsulated *Haemophilus influenzae* Strains

x	Blood and cerebrospinal fluid ^a		Nasopharynx and throat ^b	
	β -lactamase +	β -lactamase -	β -lactamase +	β -lactamase -
	3 b	31 b 1 f	10 b	33 b 1 c 12 f
		7 b	1 b	4 b
		1 b	5 c	4 c
	3	40	16	54

^a Serum strains

^b Type b strains (4 β -lactamase positive) 1 type c 12 type f and 7 type g (3 β -lactamase positive) were originally isolated strains

capsulated strains of biotype I. These were now added to biotype II as no decarboxylation of urea could be recorded. All strains were tested for porphyrins.

The strains included in the material 147 non-capsulated and 56 capsulated strains were consecutively isolated from upper respiratory tract specimens. Capsulated strains comprising 10% of consecutive isolates. Four of the 147 non-capsulated strains (2.7%) and 7 of the 56 capsulated (12.5%) produced β -lactamase ($\chi^2 = 5.78$, 1025).

DISCUSSION

Distribution of biotypes among capsulated and non-capsulated strains was in agreement with that reported by other Scandinavian authors (2, 13, 14). Further 332 capsulated strains biotyped in the present study and in the investigations cited 306 were of biotype I or IV and none of biotypes III or V. The remaining 26 strains were of biotype II. All 15 type II strains biotyped hitherto have been assigned to type IV (1, 2, 4, 13, 19).

The capacity to produce capsular polysaccharide in most *H. influenzae* strains parallels the production of both urease and ornithine decarboxylase, whereas most non-capsulated strains produce none of the enzymes.

A high proportion of biotype II among capsulated and of biotype I among non-capsulated strains reported by Albritton *et al.* may be due to ecological factors or to the inclusion of several strains from the same patient (11).

Although there is a correlation between biochemical characteristics and resistance to penicillin

of capsular polysaccharide in most strains it would not appear useful to correlate the biotypes strictly with the source of isolation as has been suggested (1, 2). Seven of 43 capsulated strains isolated from blood or cerebrospinal fluid were of biotype II in comparison with 5 of 70 capsulated strains isolated from upper respiratory tract specimens (Table 3).

Penicillin resistance in *H. influenzae* strains hitherto investigated is attributable exclusively to the presence of the TEM or type IIIa β -lactamase. The enzyme is determined by transposon A (tn A) often found on plasmids resident in gram negative bacteria (3, 6, 7, 15, 21, 22).

The incidence of penicillin resistance has been reported as 7-11% among capsulated and 1% among non-capsulated strains which is in agreement with present results (8, 11, 17, 20). The incidence of β -lactamase positive type b strains isolated from blood and cerebrospinal fluid 7.1% (Table 3) may be close to the true incidence in the population as the strains originated from severely ill patients not treated with one or more courses of antibiotics as is often the case in patients with upper respiratory tract infections before a specimen is sent for culture. This may be particularly true for capsulated β -lactamase positive strains. The significantly higher frequency of β -lactamase producers among capsulated than in non-capsulated strains from the upper respiratory tract may therefore be due to the collection of strains from selected patients though it could also indicate that capsulated strains especially type c have a greater ability to acquire plasmids carrying transposon A than non-capsulated strains have.

The significant difference in distribution of biotypes between capsulated and non-capsulated β -lactamase positive

TABLE 1 *Distribution of Biotypes among 396 Haemophilus influenzae Strains*

Biotype	Number of strains	Indole production	Urease activity	Ornithine decarboxylase
I	102	+	+	+
II	159	+	+	-
III	93	-	+	-
IV	14	-	+	+
V	25	+	-	+
Non I-V	3			

production was determined in a 24 or 48 h Levinthal broth culture with Ehrlich Bohme reagent. Urease activity was determined as described by Christensen (5). Decarboxylation of ornithine was determined according to Haller (16). Serotyping was done by slide agglutination or gel diffusion (9). β -lactamase was determined by the chromogenic cephalosporin test (10).

The criteria for *H. influenzae* were Gram negative rods or coccoid rods with growth requirement for haemin and NAD and no haemolysis on horse blood agar.

199 strains were stored prior to biotyping and 197 were biotyped when freshly isolated.

153 of the last-mentioned strains were retyped after 6-12 months of storage. The indole production was now determined with both Ehrlich Bohme and Kovacs reagents. The strains were also tested for biosynthesis of porphyrins from δ -amino-laevulinic acid in order to check the reliability of the X- and V factor disc tests (12).

RESULTS

393 of the 396 strains were assigned to biotypes I to V, while 3 strains remained unclassified (Table 1). 89% of capsulated strains produced both urease and ornithine decarboxylase and were of biotypes I or IV, while 95% of non capsulated strains belonged

to biotypes II, III and V - that is, biotypes producing either urease or ornithine decarboxylase (Table 2).

The distribution of biotypes was significantly uneven for both capsulated ($\chi^2 = 264.2$, 4 d.f., $p < 0.001$) and non-capsulated strains ($\chi^2 = 27.0$, 4 d.f., $p < 0.001$) and also significantly different between capsulated and non-capsulated strains ($\chi^2 = 277.0$, 4 d.f., $p < 0.001$).

The difference in distribution of β -lactamase positive and negative non-capsulated strains, 133 β -lactamase-positive strains of biotypes IV in contrast to 15 of 147 β -lactamase-negative strains, was statistically significant ($\chi^2 = 12.4$, 1 d.f., $p < 0.001$).

Of the 49 strains isolated from blood, cerebrospinal fluid, 43 were capsulated, 42 serotype b and 1 serotype f (Table 3). The 6 non capsulated strains isolated from blood and cerebrospinal fluid were of biotypes II (4 strains), III (1 strain) and IV (1 strain) - that is, the same biotypes as recorded for most of the non-capsulated strains isolated from the upper respiratory tract.

Retyping of 153 strains after 6-12 months preservation gave results identical with the primary typing of freshly isolated strains, except for 2

TABLE 2 *Distribution of Biotypes among Capsulated and Non-capsulated β lactamase Positive and Negative Haemophilus influenzae Strains*

Biotype	I	II	III	IV	V	Non I-V	Total
Non capsulated	11	147	93	4	25	3	283
Non-capsulated β +	0	68	45	0	20	1	134
Non-capsulated β -	11	79	48	4	5	2	149
Capsulated	91	12	0	10	0	0	113
Capsulated β +	13	1	0	5	0	0	19
Capsulated β -	78	11	0	5	0	0	94

a) β + = β lactamase positive

b) β - = β lactamase negative

ANTIBIOTIC SENSITIVITY OF ENTEROPATHOGENIC *ESCHERICHIA COLI* TO MECILLINAM, TRIMETHOPRIM-SULFAMETHOXAZOLE AND OTHER ANTIBIOTICS

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From A Antibiotic sensitivity of enteropathogenic *Escherichia coli* to mecillinam trimethoprim
sulfamethoxazole and other antibiotics. Acta path. microbiol. scand. Sect. B 88 265-268 1980

Forty-four clinical isolates of enteropathogenic *Escherichia coli* (EPEC) collected in Addis Ababa
Ethiopia 1977-1978 were tested for susceptibility to 12 different antibiotics and beta lactamase
production. Special reference was made to mecillinam and trimethoprim-sulfamethoxazole (TMP SMZ)
that were recently shown to be effective in the treatment of severe gastroenteritis caused by EPEC.
Twenty-nine of the strains were of serotype O111:B4. Thirty of the strains were resistant to 4
or more antibiotics, most of these strains belonging to serotype O111:B4. For mecillinam 19 strains
had minimal inhibitory concentration (MIC) $\leq 0.2 \mu\text{g/ml}$, 27 strains had MIC 0.8-3.2 $\mu\text{g/ml}$.
Regarding TMP SMZ 41 strains had MIC $\leq 1 \mu\text{g/ml}$, 5 strains had 2-4 $\mu\text{g/ml}$. No strain was
resistant to gentamicin or nalidixic acid. Increased production of beta lactamase was correlated to
trimethoprim resistance.

Key words: Antibiotic sensitivity, enteropathogenic *E. coli*, mecillinam, trimethoprim, sulfamethoxazole.

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Manuscript submitted 17 iv 80

The use of antibiotics in the treatment of
diarrhoea caused by enteropathogenic *Escherichia*
(EPEC) has been controversial especially in
severe cases of diarrhoea (8). Studies from
different geographical areas have advocated their
use (1, 2, 4, 9) and in consequence *in vitro*
susceptibility studies of clinical isolates to different
antibiotics have been performed (7, 10).

In the Ethio-Swedish Pediatric Clinic, Addis
Ababa, Ethiopia, we performed a controlled clinical
trial of 49 patients with severe EPEC diarrhoea.
The patients received either mecillinam
or trimethoprim-sulfamethoxazole (TMP SMZ) or no
antibiotic treatment. The clinical response was very
similar with a clinical cure rate of 79% for
mecillinam treatment and 73% for TMP SMZ
treatment and 79% for controls.

diarrhoea caused by EPEC

MATERIALS AND METHODS

Source and Identification of Bacteria

EPEC isolates from 46 patients attending the Ethio-
Swedish Pediatric Clinic, Addis Ababa, Ethiopia during
1977 and 1978 were included. Twenty-five of the
isolates belonged to patients included in the clinical trial
previously mentioned. All strains were isolated before
antibiotic therapy.

The bacteria were identified as *Escherichia coli* using
the API 20 E system (Analytab, New York, USA).
Serotyping was performed by routine methods as
previously described (12). The isolates consisted of the

tive and negative strains may indicate that non capsulated strains producing both ureas and ornithine decarboxylase are unable to acquire plasmids carrying transposon A.

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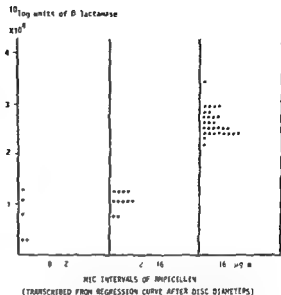
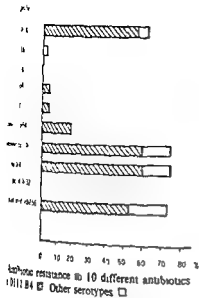


Fig 5 Beta lactamase activity related to susceptibility of ampicillin

RESULTS

Testing

Fig 1 and Fig 2 show the MIC values for ampicillin and TMP-SMZ. For mecillinam, only O111 B4 had a moderately increased MIC interval of 0.8–3.2 $\mu\text{g/ml}$ for TMP-SMZ. There was no such obvious correlation to serotype. One of the multi-resistant serotype O111 B4 was re-isolated from a patient after a 5 days course of TMP-SMZ. The MIC for TMP-SMZ had then increased from 0.8 $\mu\text{g/ml}$ before treatment to 32 $\mu\text{g/ml}$ after treatment. Fig 3 shows the number of resistant

strains to 10 other antibiotics. Multi-resistant strains generally belong to serotype O111 B4. Only 2 strains were resistant to kanamycin and neomycin, one to cephalaxin. All strains were sensitive to gentamicin and nalidixic acid.

Beta-lactamase Determination

Fig 4 and Fig 5 show the production of beta-lactamase related to the susceptibility of mecillinam and ampicillin respectively. The 27 strains of serotype O111 B4 with MIC-values for mecillinam

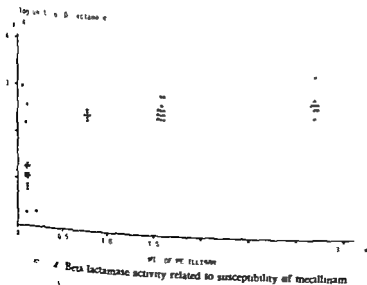


Fig 4 Beta lactamase activity related to susceptibility of mecillinam

following serotypes 044 K74 (1), 055 B5 (4), 0111 B4 (29), 0114 K90 (2), 0119 B14 (5), and 0126 B8 (2)

Assay of toxin production on all strains was performed at the National Bacteriological Laboratory, Stockholm, Sweden. No strain was toxigenic referring to heatlabile or heatstable toxin production.

Sensitivity Testing

The minimum inhibitory concentration (MIC) was determined for mecillinam and TMP-SMZ using the agar dilution method (5). Mecillinam was obtained from Leo Pharmaceutical, Copenhagen, Denmark, and TMP-SMZ from KABI, Sweden. The proportion of trimethoprim to sulfamethoxazole was 1:20, thus reflecting the serum ratio (3). *E. coli* AT CC 25922 was used as reference strain. For all other antibiotics sensitivity testing was performed according to the disc diffusion method (5). PDM medium, that was used for all determinations and antibiotic discs were purchased from Biodisk, Sweden. Breakpoints for susceptibility were in accordance to those of the Reference Group for Antibiotic Sensitivity Testing of the Swedish Medical Society's Sections for Medical Microbiology and Infectious Diseases. Breakpoints for neomycin were chosen according to Biodisk, Sweden, since neomycin is not yet included in the list of the reference group. The breakpoint for mecillinam is still only defined with reference to urinary tract infections while it is not used in this context.

Beta-lactamase Determinations

Estimations of beta lactamase activity were kindly performed by Jens Keiding, Leo Pharmaceutical Products, Copenhagen, Denmark. Estimations were carried out according to a modification of the method described by Uri *et al.* (13). Briefly, the chromogenic cephalosporin nitrocephin was used as substrate. A change of colour,

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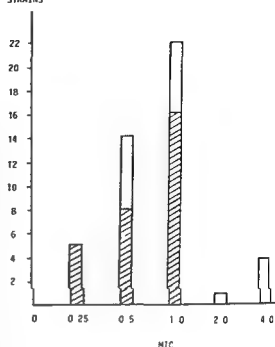


Fig. 2. MIC values for TMP/SMZ in weight proportion 1:20. Serotype 0111 B4 (hatched bars), Other serotypes (solid black bars), and unlabeled (white bars).

when the substrate was exposed to suspensions of lactamase producing bacteria, was read by visual inspection at appropriate intervals. One unit of activity defined as the amount of enzyme which in one minute volume of one ml converts the assay mixture from yellow to red.

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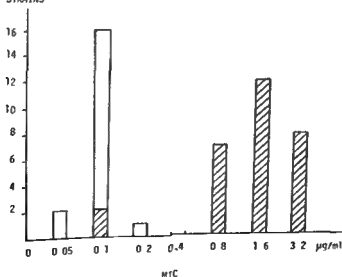
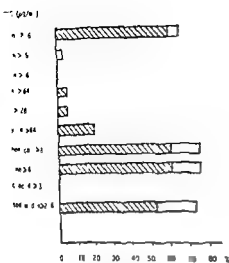


Fig. 1. MIC values for mecillinam. Serotype 0111 B4 (hatched bars), Other serotypes (white bars).



Antibiotic resistance to 10 different antibiotics
 0111 B4 Other serotypes

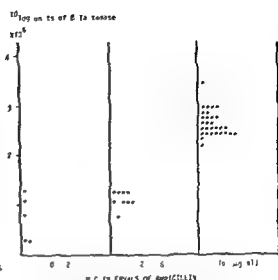


Fig 5 Beta lactamase activity related to susceptibility of ampicillin

RESULTS

In-vitro Testing

Fig 1 and Fig 2 show the MIC values for ticarcillin and TMP-SMZ. For ticarcillin only 2 of 0111 B4 had a moderately increased MIC in the interval of 0.8–3.2 μ g/ml. For TMP-SMZ, however, there was no such obvious correlation.

The MIC value for TMP-SMZ had then increased from 4 μ g/ml before treatment to 32 μ g/ml after treatment. Fig 3 shows the number of resistant strains to 10 other antibiotics. Multi-resistant strains generally belong to serotype 0111 B4. Only 2 strains were resistant to kanamycin and neomycin, one to ceftazidime. All strains were sensitive to gentamicin and nalidixic acid.

Multi-resistant strains generally belong to serotype 0111 B4. Only 2 strains were resistant to kanamycin and neomycin, one to ceftazidime. All strains were sensitive to gentamicin and nalidixic acid.

Beta lactamase Determination

Fig 4 and Fig 5 show the production of beta-lactamase related to the susceptibility of ticarcillin and ampicillin respectively. The 27 strains of serotype 0111 B4 with MIC-values for ticarcillin

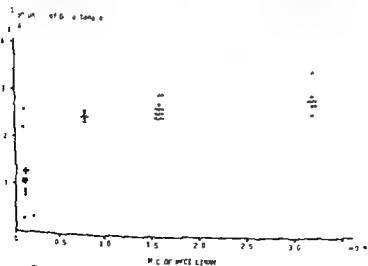


Fig 4 Beta lactamase activity related to susceptibility of ticarcillin

following serotypes 044 K74 (L) (4), 055 B5 (4), 0111 B4 (29), 0114 K90 (2), 0119 B14 (5), and 0126 B8 (2)

Assay of toxin production on all strains was performed at the National Bacteriological Laboratory, Stockholm, Sweden. No strain was toxigenic referring to heatlabile or heatstable toxin production.

Sensitivity Testing

The minimum inhibitory concentration (MIC) was determined for mecillinam and TMP-SMZ using the agar dilution method (5). Mecillinam was obtained from Leo Pharmaceutical, Copenhagen, Denmark, and TMP-SMZ from KABI, Sweden. The proportion of trimethoprim to sulfamethoxazole was 1:20, thus reflecting the serum ratio (3). *E. coli* ATCC 25922 was used as reference strain. For all other antibiotics sensitivity testing was performed according to the disc diffusion method (5). PDM medium, that was used for all determinations and antibiotic discs were purchased from Biodisk, Sweden. Breakpoints for susceptibility were in accordance to those of the Reference Group for Antibiotic Sensitivity Testing of the Swedish Medical Society's Sections for Medical Microbiology and Infectious Diseases. Breakpoints for neomycin were chosen according to Biodisk, Sweden, since neomycin is not yet included in the list of the reference group. The breakpoint for mecillinam is still only defined with reference to urinary tract infections while it is not used in this context.

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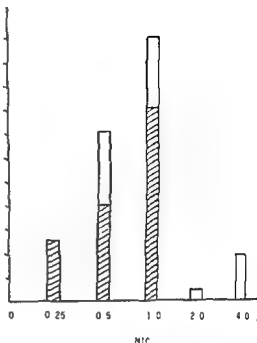


Fig. 2 MIC values for TMP/SMZ in weight proportion 1:20. Serotype 0111 B4 (hatched bars) Other serotypes (white bars).

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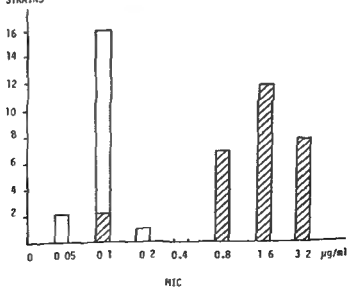


Fig. 1 MIC values for mecillinam. Serotype 0111 B4 (hatched bars) Other serotypes (white bars).

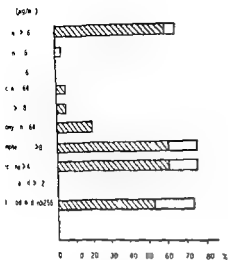


Fig 3 Antibiotic resistance to 10 different antibiotics (0111 B4) Other serotypes

RESULTS

Sensitivity Testing

Fig 1 and Fig 2 show the MIC values for ampicillin and TMP SMZ. For ampicillin only strains of 0111 B4 had a moderately increased MIC in the interval of 0.8-32 µg/ml. For TMP SMZ however there was no such obvious correlation of MIC to serotype. One of the multi-resistant strains of serotype 0111 B4 was isolated from a patient after a 5 days course of TMP SMZ. The MIC value for TMP SMZ had then increased from 0.8 µg/ml before treatment to 32 µg/ml after treatment. Fig 3 shows the number of resistant

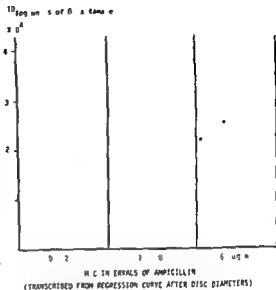


Fig 5 Beta-lactamase activity related to susceptibility of ampicillin

strains to 10 other antibiotics. Multi-resistant strains generally belong to serotype 0111 B4. Only 2 strains were resistant to kanamycin and neomycin, one to cephalexin. All strains were sensitive to gentamicin and nalidixic acid.

Beta-lactamase Determination

Fig 4 and Fig 5 show the production of beta-lactamase related to the susceptibility of ampicillin and ampicillin respectively. The 27 strains of serotype 0111 B4 with MIC-values for ampicillin

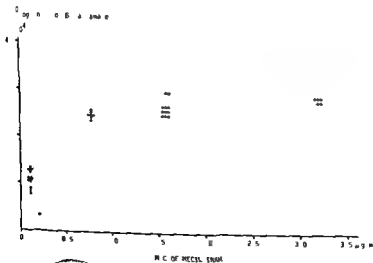


Fig 5 Beta-lactamase activity related to susceptibility of ampicillin

in the interval of 0.8–3.2 µg/ml all had increased beta lactamase activity (Fig. 4). However 3 strains with high beta lactamase activity had low MIC-values for mecillinam (0.05–0.1 µg/ml). In contrast Fig. 5 demonstrates that all strains with high beta-lactamase activity were resistant to ampicillin.

DISCUSSION

The treatment of widespread and potentially serious infections such as EPEC gastroenteritis poses some considerations concerning an 'ideal' antibiotic: high efficacy against the offensive organism, low toxicity, low incidence of plasmid mediated R factors and (especially in developing countries) low cost. Aminoglycosides and colistin have been widely used as effective drugs for *E. coli* infections although they may have some toxic effects. Regarding ampicillin, sulfonamides, tetracycline and chloramphenicol, widespread resistance of EPEC strains has been reported (1, 2, 4, 7, 10).

Mecillinam and TMP SMZ are 2 relatively new drugs reported to have high efficacy against *E. coli* infections and low toxicity (3, 6). These 2 drugs were successfully used in the treatment of severe EPEC gastroenteritis (12) and *in vitro* investigation of susceptibility confirmed that the MIC values for mecillinam were 5–10 times below the serum concentrations usually obtained with the given dosages of the drug (40 mg per kg of bodyweight i.m.) (11). A moderate increase of MIC values to mecillinam was always associated with ampicillin resistance and confined to the most frequent serotype (O111:B4). It is not clear whether increased MIC for mecillinam depends on increased beta lactamase activity, since the few ampicillin resistant strains of other serotypes with increased beta lactamase activity as well had low MIC values for mecillinam. The susceptibility of these isolates to TMP SMZ was generally good. However the marked increase of MIC in one of the strains after treatment with TMP SMZ is an observation.

This investigation shows that clinical EPEC isolates from Ethiopia are frequently multi resistant: many of the isolates belonging to serotype O111:B4. Modern drugs such as mecillinam and TMP SMZ should be considered as alternatives beside established treatment like aminoglycosides.

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EXPERIMENTAL ENDOCARDITIS IN RABBITS

1 Experiments with *Serratia marcescens* on the Significance of Serum Susceptibility and Proteolytic Capacity of the Strains and the Influence of an Indwelling Catheter

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Gutschik, E., Norwood, R. S., Möller, S. & Olling, S. Experimental endocarditis in rabbits. 4
Experiments with *Serratia marcescens*. On the significance of serum susceptibility and proteolytic
capacity of the strains and the influence of an indwelling catheter. Acta path. microbiol. scand. Sect. B
88 269-276 1980.

In order to investigate the course of *Serratia marcescens* endocarditis in groups of rabbits with and without an indwelling catheter 130 rabbits were pretreated to produce left-sided endocarditis. Three clinical isolates of *S. marcescens* were used to infect the rabbits i.e. CDC 013 (serum sensitive proteolytic) SMI 104 (serum resistant proteolytic) and SMI 55 (highly serum resistant non proteolytic). Ten rabbits with an indwelling catheter were challenged with CDC 013 and none of them died or showed evidence of endocarditis 28 days later. In groups of rabbits with indwelling catheters which were challenged with SMI 104 or SMI 55 there was a high incidence of endocarditis (19/20 18/20 respectively) while groups without catheters inoculated with the same strains had a lower incidence (5/20 15/20 respectively). In contrast to earlier observations with *Streptococcus faecalis* the clinical and pathological data were not significantly influenced by the presence or absence of proteolytic capacity of the infecting strains. The results indicate that the ability of *S. marcescens* to establish endocarditis depends significantly on the degree of serum resistance of the strains. This difference was only demonstrable in experiments without an indwelling catheter during the infection period. The disturbing influence of an indwelling catheter is discussed and it is concluded that experimental models using indwelling catheters are inappropriate for studies on the pathophysiology of endocarditis.

Key words: Experimental endocarditis, rabbits, *Serratia marcescens*, proteolytic capacity, serum bacterial activity, indwelling catheter.

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Serratia marcescens is usually considered to be a species with low pathogenicity for man, although it is frequently isolated from the urinary and respiratory tracts. Nevertheless, endocarditis caused by *S. marcescens* has been described with increasing frequency (5), especially in drug addicts (12). Furthermore, wound and blood stream infections when they do occur, are associated with high death rates (17, 18). The importance of left

sided valvular involvement in *Serratia* endocarditis is interesting because it is in contrast to the predominantly right sided location of *Staphylococcus aureus* endocarditis in addicts. *Serratia* endocarditis does however in its location resemble the enterococcal endocarditis (3) produced in the rabbit model described earlier (9, 10).

The clinical and pathological features and variations in the course of both spontaneous and experimental endocarditis are in part explained by

the influence of one or more bacterial virulence factors such as the ability of the bacteria to adhere to the endocardium (14) their proteolytic capacity (10) and their resistance or sensitivity to bactericidal serum factors (1-4)

In order to learn more about the host parasite relationship in cases of *S. marcescens* endocarditis we carried out a series of experiments using our model of experimental rabbit endocarditis (8-9). The aim was to study the course of untreated *S. marcescens* endocarditis in cases with and without a retained catheter and after inoculation with strains differing in serum susceptibility and proteolytic capacity.

MATERIALS AND METHODS

Strains

Three clinical isolates of *S. marcescens* identified by routine laboratory tests and with the following common characters were used: Gram negative motile rods, indole negative, Voges-Proskauer positive, DNAse positive, ornithine and lysine decarboxylase positive and able to reduce nitrate to nitrite. They produced acid but no gas in glucose and sucrose and did not ferment lactose. The antibiotic susceptibility pattern showed resistance to ampicillin, cephalothin, polymyxin and chloramphenicol and susceptibility to gentamicin, kanamycin and carbenicillin. The individual strains were characterized as follows:

Strain CDC O13 was non-pigmented, liquefied gelatin (7) and fibrin (2) within 24 hours and had the serotype designation O13:H₄ (this strain was received by the courtesy of Professor Walter H. Traub, Institut für Hygiene und Mikrobiologie der Universität des Saarlandes, Homburg, W. Germany).

Strain SM 55 was non-pigmented and did not liquefy gelatin or fibrin.

Strain SM 104 was pigmented and liquefied gelatin and fibrin within 24 hours.

Test for fibrinolysis was carried out on a fibrin plate (2) inoculated with a drop of heavy suspension of bacteria. Incubation was at 35 °C and the last reading was recorded after 3 days. Liquefaction around the inoculum was evidence of a positive reaction.

broth (BHI broth, Difco Laboratories) cultures of the strains were grown overnight at 37 °C and then diluted 1:100 in Hanks saline. For each strain pairs of tubes each with 0.1 ml rabbit serum were prepared: one tube with fresh serum and the other with heat-inactivated serum. To each tube 0.1 ml of a bacterial

suspension adjusted at 37 °C to a density of 2.20×10^8 c.f.u./ml in roth chilled at 4 °C was added to each tube.

overnight all tubes were reincubated 4-5 hours and then agitated and placed directly in a Vitatron photometer. The optical density (597 nm) of each tube was recorded.

Quantitative blood culture was performed as described earlier (10). Blood specimens were taken every 2 weeks during the first 2 weeks after infection and three times weekly in the following weeks until the animals died spontaneously or were killed 28 days after challenge.

Experimental Groups

Randomly bred male albino rabbits (Ssc CPH) from Statens Serum Institut with an average weight of 300 (SD 123) and an average age of 181 days (SD 30) were used.

Group I Endocarditis was induced in 80 rabbits with an indwelling catheter during the experimental period (Table 1).

Group II Endocarditis was induced in 50 rabbits without an indwelling catheter during the experimental period (Table 1).

Group III Nine healthy non-operated rabbits were investigated for bacterial clearance after injection of approximately 10^8 bacteria intravenously. Three rabbits were challenged with strains CDC O13, SM 55 and SM 104 respectively. From these animals blood samples were taken for quantitative blood culture 5, 10, 20, 30, 60 and 120 min after challenge and then daily for 8 days after which the rabbits were sacrificed and autopsied.

Pretreatment for Producing Left-sided Sterile Endocarditis

The rabbits were anaesthetized i.v. with mebum sodium. The average dose at the first operation was 2.1 mg/kg (SD 1.62 (130 observations)) and at the second operation 2.32 mg/kg (SD 2.1 (49 observations)). A polyethylene catheter was inserted into the left ventricle of the heart using the technique described previously (1). The catheter was either left *in situ* for 3 days or retained for the entire experimental period.

Infection of Pretreated Animals

After 3 days with the catheter *in situ* the rabbits of experimental group II were subjected to a second operation involving the removal of the catheter and simultaneous injection of 1 ml suspension of *S. marcescens* into a marginal ear vein. The rabbits of experimental group I with retained catheter were challenged in a similar way. The bacterial suspension in saline was prepared from an overnight culture grown on 5% blood agar. This suspension contained approximately 10^8 colony forming units (CFU) per ml (mean 1.74×10^8 , range $0.74-3.67 \times 10^8$).

Autopsy

Autopsy was carried out as described earlier (11). Bacteria recovered from blood, vegetations and different organs were identified by their characteristic appearance on blood agar and by microscopy of Gram-stained slides. Semiquantitative culture was made from the peritoneal cavity, liver, spleen, kidneys, mesenteric glands and pleural cavity.

hearts of five rabbits (two from rabbits infected 155 two from rabbits infected with SM 104 all dwelling catheter and one heart from a rabbit with SM 104 but without indwelling catheter) used exclusively for histological examination as earlier (10). The gross structure of liver mesenteric glands and suprarenal glands was of infarcts in the kidneys described and counted in surface extension measured.

Statistical Methods

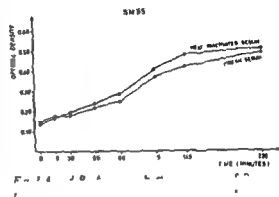
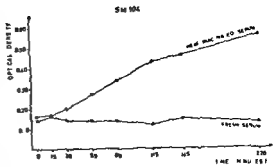
Results from the tests for serum susceptibility of SM 104 were examined by means of analysis of variance, testing the significance of the slopes. The curves for SM 55 in fresh and inactivated sera compared using a two-way analysis of variance. A comparison was also employed for the comparison of results showing the in vivo clearance of bacteria. A comparison between incidence of endocarditis was by Fisher's exact test which was also used for the incidence of early death defined as survival time less than 12 days in groups of rabbits with without an indwelling catheter. All means given in mm geometric and the standard deviations are in per cent of the means. Weights of spleen, heart

and vegetations together without taking into account whether they died spontaneously or were sacrificed on 14th day. This was the case for the variables weight of spleen and heart, but not of vegetations and CFU/g tissue. When the results from rabbits with infected vegetations to SM 55 and SM 104 were compared within each of the two experimental subgroups and the variation between the two subgroups and between rabbits with and without infected vegetations was examined.

RESULTS

Sensitivity of the Infecting Strains to the Bactericidal Action of Normal Serum

In the photometric growth assay (test A) CDC O13 was found to be markedly sensitive to three normal human sera and 20 normal rabbit sera. The SM 55 and SM 104 were classified as serum resistant. In order to further examine the resistance of SM 55 and SM 104 an assay by bacterial growth in serum (test B) was carried out. Fig. 1 A and B shows clearly that strain SM 55 gave good growth in both heat inactivated serum and fresh rabbit serum, though significantly weaker in the latter than SM 104 grew only in inactivated serum during the 220 min incubation period. These results indicate that SM 55 was significantly more serum resistant than SM 104.



different time periods as indicated.

Clearance of the Different Strains of *S. marcescens* in Non-treated Rabbits

Fig. 2 shows that after i.v. challenge of rabbits the elimination of the organisms was most rapid for the serum sensitive strain CDC O13 and that the moderately serum resistant strain SM 104 was cleared more rapidly than the highly serum resistant

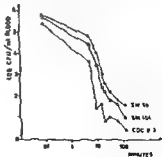


Fig. 2 Bacterial clearance of *Serrana marcescens* strains. Three rabbits were challenged i.v. (1 ml of a suspension of approximately 10^8 bacteria/ml range $1.25-1.77 \times 10^8$) with strains SM 55, SM 104 and CDC O13 respectively. Blood samples were taken after 5 seconds, 5, 10, 15, 20, 30, 60 and 120 min (log scale) as indicated.

TABLE 1 Incidence (Ratio Infected Challenged Rabbits) of Experimental *Serratia marcescens* Endocarditis in Rabbits at Indicated Time after Challenge

Rabbit group	Infecting strain	Rabbits sacrificed after			Rabbits dead spontaneously before day 28	Rabbits sacrificed after day 28	Total
		1 day	2 days	3 days			
I Catheter in situ	SM 55	5/5	5/5	4/5	12/12	6/8	18/20
	SM 104	5/5	5/5	5/5	15/15	4/5	19/20
	CDC O13	ND	ND	ND	None	0/10	0/10
II Catheter removed	SM 55	3/5	ND	ND	11/11	4/9	15/20
	SM 104	3/5	ND	ND	4/4	1/16	5/20

ND = not done

strain SM 55. The significant differences are apparent after 5 min and then persist practically unaltered during the 120 min observation period.

Incidence of Endocarditis Related to Presence or Absence of Catheter and Characteristics of the Infecting Strains

Left sided sterile endocarditis was induced in 130 rabbits. The establishment of bacterial endocarditis after challenge was confirmed by isolation of *S. marcescens* from the vegetations or by histological examination. In group I a high incidence of bacterial endocarditis was recorded in rabbits infected with the highly serum resistant strain SM 55 and with the moderately serum resistant strain SM 104 while none of the rabbits challenged with the serum sensitive strain CDC O13 developed infection ($p < 0.001$) (Table 1). In group II the incidence of bacterial endocarditis was 3 out of 5 due to both SM

55 and SM 104 one day after challenge. However, in rabbits observed for 28 days a significantly lower incidence ($p = 0.004$) of bacterial endocarditis occurred due to the moderately serum resistant strain SM 104 than due to the more resistant strain SM 55. The different incidence of endocarditis due to SM 104 in groups I and II is highly significant ($p < 0.001$), whereas the incidence of SM 55 infections was not significantly different in the groups.

Survival Time Related to Presence or Absence of Catheter

Fig. 3 shows the survival times of rabbits died spontaneously of endocarditis and the number of surviving rabbits sacrificed 28 days after challenge. The number of early deaths (*i.e.* survival time < 12 days) is significantly higher in rabbits with an indwelling catheter (14/27) than in those lacking a catheter (1/15) ($p < 0.001$). Most of the rabbits with an indwelling catheter which survived for 28 days still had infected vegetations, while most of those without a retained catheter had sterile vegetations or macroscopically normal valve leaflets. This is particularly true for rabbits challenged with the moderately serum resistant strain SM 104.

Appearance, Weight and Bacteriology of Vegetations

Distribution of vegetations was different in rabbits with and without an indwelling catheter during the experimental period. Rabbits without a catheter had their vegetations chiefly on the aortic valve leaflets, while rabbits with an indwelling catheter also had large vegetations outside the aortic valve leaflets, *i.e.* on the wall of the aorta and along the catheter on the wall of the left ventricle. It was a regular feature that only one or two of the three aortic valve leaflets was affected and that the vegetations remained attached to the origin of the

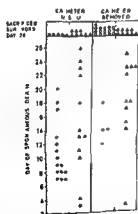


Fig. 3 Survival time of rabbits with *Serratia marcescens* endocarditis. Each dot represents one rabbit inoculated with SM 104 (● infected vegetations, ○ non infected vegetations) or with SM 55 (▲ infected vegetations, △ non infected vegetations).

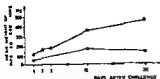


Fig. 4. Mean weight (mg) of infected vegetations in rabbits succumbed spontaneously or sacrificed at the time indicated. The results for the non-sacrificed groups are plotted against the mean survival time 12.4 days. Δ rabbits with an indwelling catheter. \circ rabbits without an indwelling catheter.

Tested area. No dissimilarities were observed in the consistency or friability of vegetations infected with different strains. Visible destruction of valve leaflets was not recorded.

Vegetations were found in all rabbits with an indwelling catheter but only in 23/50 of those without. All rabbits challenged with the serum sensitive strain CDC O13 had sterile vegetations mean weight 76.2 mg (SD 89%). The weight of infected vegetations was found to be significantly higher in rabbits with an indwelling catheter at all times during the post-exposure period than in rabbits where the catheter had been removed (Fig. 4). There was no significant difference between the weights of vegetations in rabbits infected with SM 55 and in those infected with SM 104. The bacterial density in the vegetations of rabbits that spontaneously succumbed to endocarditis was high. The mean number of CFU in rabbits with an indwelling catheter was $10^{8.9}$ /g vegetation and in those without it was $10^{10.7}$ /g. The level of CFU in the vegetations was not significantly different in rabbits

inoculated with SM 55 and with SM 104. Surviving rabbits sacrificed on day 28 of the experimental period showed considerable variation in the number of CFU indicating different stages in the infectious process (Fig. 5). The mean number of CFU/g vegetation on days 1, 2 and 3 after challenge was $10^{6.75}$, $10^{7.7}$ and $10^{9.00}$, respectively in rabbits with an indwelling catheter, indicating the rapid development of the infection. In rabbits without an indwelling catheter a result of $10^{4.98}$ CFU/g vegetations was recorded on day 1 after challenge i.e. significantly different ($p < 0.005$) from the level of CFU in rabbits with an indwelling catheter.

Histopathology of Vegetations

The vegetations consisted of an amorphous eosinophilic mass with dispersed single granulocytes mononuclear cells and aggregates of bacteria occurring as oval or circular rings. The density of bacteria observed was very high in all vegetations on the aortic valve leaflets. From vegetations outside the aortic valves the foci of bacteria were small or lacking and the tendency towards endothelialization was usually most pronounced here. No clear-cut histological differences could be observed between vegetations infected with the proteolytic strain SM 104 and the non proteolytic strain SM 55.

Quantitative Blood Culture

The nine non-operated rabbits in group III were observed for circulating bacteria in the blood stream 1 to 9 days after challenge by taking daily blood specimens. None of the rabbits inoculated with the serum sensitive strain CDC O13 had positive blood cultures. In the majority of rabbits inoculated with SM 55 and SM 104 positive blood cultures but only few bacteria per ml were observed during the first 4 days after challenge but not later.

From the rabbits pretreated for endocarditis 47.3% of 1354 blood specimens were bacteriologically positive. None of the rabbits inoculated with CDC O13 had positive blood cultures during the 28 days of the experimental period. In rabbits with an indwelling catheter inoculated with SM 55 or SM 104 positive blood samples were recorded in 51.6% (261/506) while in catheter free rabbits only 38.2% (230/608) were positive. Furthermore rabbits with an indwelling catheter that died spontaneously of endocarditis had more positive blood cultures (197/290 ~ 67.9%) than those without a catheter (170/287 ~ 59.2%). Significant differences could not be demonstrated between groups of rabbits inoculated with SM 55 and SM 104.

The level of CFU/ml blood was between 10 and 100 in the majority of positive blood cultures. High numbers of bacteria i.e. at the level of 10^3 or 10^4 /

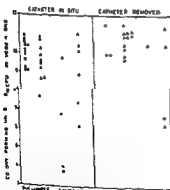


Fig. 5. Number of CFU/g blood over 28 days after challenge.

TABLE 1 Incidence (Ratio Infected Challenged Rabbits) of Experimental *Serratia marcescens* Endocarditis in Rabbits Indicated Time after Challenge

Rabbit group	Infecting strain	Rabbits sacrificed after			Rabbits dead spontaneously before day 28	Rabbits sacrificed after day 28	Total
		1 day	2 days	3 days			
I Catheter in situ	SM 55	5/5	5/5	4/5	12/12	6/8	18/20
	SM 104	5/5	5/5	5/5	15/15	4/5	19/20
	CDC O13	ND	ND	ND	None	0/10	0/10
II Catheter removed	SM 55	3/5	ND	ND	11/11	4/9	15/20
	SM 104	3/5	ND	ND	4/4	1/16	5/20

ND = not done

strain SM 55. The significant differences are apparent after 5 min and then persist practically unaltered during the 120 min observation period.

Incidence of Endocarditis Related to Presence or Absence of Catheter and Characteristics of the Infecting Strains

Left-sided sterile endocarditis was induced in 130 rabbits. The establishment of bacterial endocarditis after challenge was confirmed by isolation of *S. marcescens* from the vegetations or by histological examination. In group I a high incidence of bacterial endocarditis was recorded in rabbits infected with the highly serum resistant strain SM 55 and with the moderately serum resistant strain SM 104, while none of the rabbits challenged with the serum sensitive strain CDC O13 developed infection ($p < 0.001$) (Table 1). In group II the incidence of bacterial endocarditis was 3 out of 5 due to both SM

55 and SM 104 one day after challenge. However, after 2 and 3 days the incidence of endocarditis was significantly higher in rabbits with an indwelling catheter than in those without a catheter.

104 than due to the more resistant strain SM 55. The different incidence of endocarditis due to SM 104 in groups I and II is highly significant ($p < 0.001$), whereas the incidence of SM infections was not significantly different in the two groups.

Survival Time Related to Presence or Absence of Catheter

Fig. 3 shows the survival times of rabbits that died spontaneously of endocarditis and the number of surviving rabbits sacrificed 28 days after challenge. The number of early deaths (i.e. survival time < 12 days) is significantly higher in rabbits with an indwelling catheter (14/27) than in rabbits lacking a catheter (1/15) ($p < 0.001$). Most of the rabbits with an indwelling catheter which survived for 28 days still had infected vegetations, while most of those without a retained catheter had sterile vegetations or macroscopically normal valve leaflets. This is particularly true for rabbits challenged with the moderately serum resistant strain SM 104.

Appearance, Weight and Bacteriology of Vegetations

Distribution of vegetations was different in rabbits with and without an indwelling catheter during the experimental period. Rabbits without a catheter had their vegetations chiefly on the aortic valve leaflets, while rabbits with an indwelling catheter also had large vegetations outside the aortic valve leaflets, i.e. on the wall of the aorta and also on the catheter on the wall of the left ventricle. It was a regular feature that only one or two of the three aortic valve leaflets was affected and that the vegetations remained limited to the origin:

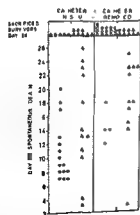


Fig. 3 Survival time of rabbits with *Serratia marcescens* endocarditis. Each dot represents one rabbit inoculated with SM 104 (● infected vegetations, ○ non infected vegetations) or with SM 55 (▲ infected vegetations, △ non infected vegetations).

Our results are in agreement with observations in human infections which show that traumatic *S. marcescens* strains are invariably resistant (15) although delayed serum neutralizing strains isolated from the blood stream have been described recently (16). The influence of catheters was emphasized by Wilfert et al. (17) who found that in 44% of 39 episodes of an *S. marcescens* bacteraemia the indwelling catheter was the probable portal of entry. The presence of an indwelling catheter in the experimental model led to continuous enlargement of vegetations not only at the aortic valve but also along the catheter below and above the valves (8). The histological appearance of the vegetations in the rabbits pretreated for endocarditis however is different on the aortic leaflets and outside these as earlier described indicating that bacterial aggregation and that in these two types of vegetations might be different. Later investigations of the spontaneous infection of *S. faecalis* (9, 10) and the present investigation of *S. marcescens* endocarditis seem to confirm these early observations. The histological examinations showed that the density of bacteria is very high in all vegetations on the aortic valve but only small foci of bacteria were found on the valves. Quantitative cultures showed ten to several hundred times difference in bacterial density in these two types of vegetations (unpublished work). Similar observations were published recently by Francioli & Freedman (6) who compared the bacterial density of *Streptococcus sanguis* in the vegetations of aortic and tricuspid valves with vegetations in different parts of the vascular system of the heart. The lower mean number of CFU/

ment of small new vessels and proliferating fibroblasts and probably also fibrinolytic action on the vegetations by serum factors as described earlier (8). The extent of these latter processes is underlined by the surprisingly rapid disappearance (4-10 days) of the sterile vegetations developed after 3 days with catheter *in situ*.

The role of the indwelling catheter during the infectious process is not limited to its mechanical effect. It probably influences the physiological processes in its proximity thereby upsetting the balance between the forces involved and resulting in delayed recovery. Our results demonstrate that an indwelling catheter will affect both the frequency of infection establishment and the outcome of the endocarditis. The higher incidence of endocarditis 1 day after challenge, the higher weight of vegetations and the higher number of CFU/g vegetation in rabbits with an indwelling catheter compared with those without a catheter may be due to a more rapid covering of both bacteria and catheter with fibrin which affords protection against humoral and cellular defence mechanisms. These physiological changes induced by the catheter are probably also responsible for the significantly higher incidence of early death in rabbits with an indwelling catheter. Thus the course of infection will be acute and severe.

Altogether the results show that an indwelling catheter contributes to the maintenance of an infection which would take a significantly different course without the catheter depending on the degree of serum resistance of the infecting strains. Therefore it is our opinion that an experimental model using an indwelling catheter is not ideal and should not be used indiscriminately.

We are indebted to Dr N Christensen, Department of Pathology, Frederiksberg Hospital, Copenhagen for preparing the histological slides of endocardial vegetations of rabbit hearts and K L Fennestad, V M D, Statens Seruminstitut, for provision and maintenance of the rabbits. We thank Mrs U A S Petersen and Miss K Florén for technical assistance.

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ml blood occurred intermittently, such values were without predictive importance for the time of spontaneous death. The level of bacteraemia in rabbits which died spontaneously of endocarditis was slightly elevated terminally, but there were no signs of an overwhelming generalized infection.

Spleen

In 33 rabbits without evidence of endocarditis (28 days observation), the mean weight of the spleens was 1.18 g (SD 35%), i.e. significantly lower than in 57 rabbits with infected vegetations (2.39 g, SD 83%). No significant differences occurred between the spleen weights of rabbits inoculated with SM 55 (mean 2.36 g) and SM 104 (mean 2.41 g). A spleen weight >2 g was found in 34/57 rabbits (59.6%) with infected vegetations, but only in 1/33 rabbits without signs of endocarditis. Splenic infarction was recognized in 3/130 rabbits.

Kidneys

In rabbits with an indwelling catheter and evidence of bacterial endocarditis, 69 out of 78 kidneys (88%) had one or more infarcts, while in rabbits lacking an indwelling catheter, 24 out of 40 kidneys (60%) had infarcts. There was no significant difference between groups of rabbits infected with SM 55 and with SM 104. The incidence of large infarcts (≥ 10 mm²) was slightly higher in rabbits infected with SM 104 (34/41 infarcted kidneys) than in those infected with SM 55 (33/51).

Other Organs

The weights of the hearts were not significantly different in rabbits with infected vegetations due to SM 55 and SM 104 (28 days observation). The mean weight of hearts in all rabbits with evidence of endocarditis was 9.23 g (SD 24%, 52 observations), while in all rabbits without evidence of endocarditis it was 8.30 g (SD 24%, 32 observations).

Focal infectious processes (except for the kidneys) yielding growth of *S. marcescens* were seen as subphrenic abscesses or abscesses in the liver in 4/130 rabbits. Three rabbits had neurological symptoms with paralysis of their legs. Pericardial effusion of 2–5 ml was found in 50/130 rabbits while pleural and peritoneal effusions more than a few ml were seldom recorded.

Rabbits with heavily infected vegetations ($\geq 10^9$ CFU/g) nearly always had positive blood cultures terminally and also heavy growth of *S. marcescens* from the spleen, liver, kidneys and, to a smaller extent, from the lungs and mesenteric glands. On the other hand, rabbits with slightly infected vegetations ($<10^9$ CFU/g) had negative blood cultures terminally and no growth from the organs.

DISCUSSION

The three *S. marcescens* strains used to produce endocarditis varied in two characters: degree of serum susceptibility and proteolytic capacity. However, pathological and clinical data indicated a separate effect due to the proteolytic capacity of the strains were not obtained. This was:

since it had earlier been shown by us (10) that proteolytic strains of *Streptococcus faecalis* produced a more severe and acute course of endocarditis characterized by a shorter survival time and a higher degree of bacteraemia with small amounts of soft, friable vegetations in the left side of the heart and a large number of kidney infarcts than non-proteolytic strains, probably as a result of the dissolution of fibrin in the vegetations. Why the very strong *in vitro* proteolytic activity of the *marcescens* strain SM 104 was not apparent *in vivo* conditions is not clear, theoretically it might be due to a blocked production of proteases inside the vegetations or inhibition of native protease inhibitors of rabbit plasma. The latter possibility is supported in the work of Hochstrasser *et al.* (11) where it is shown that the proteases of *Pseudomonas aeruginosa* strains are practically completely inhibited by human and animal α_2 -macroglobulin.

The different rates of infection and spontaneous death observed in our experimental model of *marcescens* endocarditis appear to be due to the effect of the serum bactericidal system. In rabbits with an indwelling catheter none of the animals could be infected with the serum sensitive strains whereas nearly all rabbits were infected with moderately and the highly serum resistant strains. This is in accordance with Archer & Fekety's observation that serum sensitive strains of *Pseudomonas aeruginosa* failed to infect sterile vegetations in their rabbit model of endocarditis using an indwelling catheter during the experimental period. Similarly, using seven serum resistant and seven serum sensitive strains of *Escherichia coli* Dura & Beeson (4) showed that the ability of the strains to establish endocardial infections was directly associated with the resistance to the complement mediated serum bactericidal system. Experiments with 11 serum resistant *S. marcescens* strains showed that rabbits infected with a highly resistant strain had significantly higher incidence of endocarditis (75%) than those infected with the moderately serum resistant strain (25%). Such a difference could only be demonstrated in rabbits with no indwelling catheter during the experimental period and therefore an indwelling catheter must be assumed to be able to camouflage minor differences in serum susceptibility.

results are in agreement with observations in human infections which show that *S. marcescens* strains are invariably resistant (15) although delayed serum conversion strains isolated from the blood-stream described recently (16). The influence of indwelling catheters was emphasized by Wilfert *et al.* (17) who found that in 44% of 39 episodes of *S. marcescens* bacteraemia the indwelling catheter was the probable portal of entry.

The presence of an indwelling catheter in the experimental model led to continuous enlargement of vegetations not only on the aortic valve but also along the catheter below and above the valve (8). The histological appearance of the vegetations in the rabbits pretreated for endocarditis however is different on the aortic leaflets and outside these as earlier described indicating that bacterial aggregation and infection in these two types of vegetations might be different.

Later investigations of the spontaneous endocarditis of *S. faecalis* (9, 10) and the present investigation of *S. marcescens* endocarditis seem to confirm these early observations. The histological examinations showed that the density of bacteria was very high in all vegetations on the aortic valve but only small foci of bacteria were found on the valves. Quantitative cultures showed ten to several hundred times difference in bacterial density in these two types of vegetations (unpublished work). Similar observations were published by Francioli & Freedman (6) who compared the bacterial density of *Streptococcus sanguis* in the vegetations on aortic and tricuspid valves with vegetations in different parts of the vascular system of the heart. The lower mean number of CFU/vegetation found in our study in rabbits with an indwelling catheter compared with those without a catheter may be a consequence of the practice of sampling and pooling all vegetations from an animal in this way vegetations from different sites with different bacterial density will influence the mean value.

The role of the catheter during pretreatment (8, 9) is probably mechanical. The later adhesion of bacteria to vegetations and the development of infection take place without the presence of the catheter. Bacteria act as small foreign bodies and their presence will lead to continued deposition of fibrin and thrombocytes thus increasing the size of the vegetations. The actual extension of vegetations may be the result of a subtle balance between forces favouring a deposition of fibrin and thrombocytes and other forces provoking a diminution of the vegetations such as mechanical separation of small clots or organization of the vegetations by develop-

ment of small new vessels and proliferating fibroblasts and probably also fibrinolytic action on the vegetations by serum factors as described earlier (8). The extent of these latter processes is underlined by the surprisingly rapid disappearance (4-10 days) of the sterile vegetations developed after 3 days with catheter *in situ*.

The role of the indwelling catheter during the infectious process is not limited to its mechanical effect. It probably influences the physiological processes in its proximity, thereby upsetting the balance between the forces involved and resulting in delayed recovery. Our results demonstrate that an indwelling catheter will affect both the frequency of infection establishment and the outcome of the endocarditis. The higher incidence of endocarditis 1 day after challenge, the higher weight of vegetations and the higher number of CFU/g vegetation in rabbits with an indwelling catheter compared with those without a catheter may be due to a more rapid covering of both bacteria and catheter with fibrin which affords protection against humoral and cellular defence mechanisms. These physiological changes induced by the catheter are probably also responsible for the significantly higher incidence of early death in rabbits with an indwelling catheter. Thus the course of infection will be acute and severe.

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THE GLUTARALDEHYDE-FIXED CYTOCENTRIFUGE- PREPARED URINE SEDIMENT IN THE EVALUATION OF URINARY TRACT INFECTION

The Significance of Pyuria

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Norberg B, Norberg A & Gippert H. The glutaraldehyde fixed cytocentrifuge prepared urine sediment in the evaluation of urinary tract infection. The significance of pyuria. Acta path microbiol Scand Sect B 88: 277-279, 1980.

During antibiotic treated urinary tract infection in non-catheterized geriatric inpatients bacteriuria appeared within 1-2 days. Pyuria on the other hand disappeared within 4-7 days. After withdrawal of antibiotic therapy bacteriuria reappeared quicker than pyuria. It is suggested that the time lag between bacteriuria and pyuria is due to the fact that bacteriuria reflects bacterial invasion into the urinary tract whereas pyuria reflects the inflammatory response of the urinary tract.

Keywords: Urinary tract infection, bacteriuria, pyuria, urine sediment.

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Conventional urine sediment is distinguished by its unreliability and poor validity. The back-
ground of this nuisance is complex. The main factors which invalidate the urine sediment are varying content in the urine, bacterial growth after sampling, cell and cast disintegration after sampling, varying volume of resuspension, urine volume of resuspended urine droplet on the poor contrast of specimens and poor microscopic resolution (1-9).

We have tried to offset the weaknesses of the conventional urine sediment by the development of a quantified (PQ) urine preparation. This method was distinguished by standardized sampling, immediate fixation with glutaraldehyde, cytocentrifugation and contrasting with routine methods (5, 6, 7).

In the evaluation of urinary tract infection (UTI) there has been a matter of dispute (1, 3, 4, 9). The study was designed to elucidate the course

and role of pyuria in bacterial UTIs of non-catheterized geriatric inpatients by means of the PQ urine sediment.

MATERIAL AND METHODS

This study was conducted on 30 consecutive non-catheterized inpatients from the psychogeriatric wards of Saint Lars Hospital in Lund. The patients were admitted to the Department of Internal Medicine because of clinical symptoms of UTI, bacteria and/or leukocytes in a conventional urine sediment and a positive routine quantitative urine culture. Prior to the urine sampling the orificium of the urethra was cleaned with sterile saline. The control samples prior to antibiotic treatment were delivered as two non-morning midstream urine samples with an interval of two hours. The urine was collected in a sterile plastic tube and thoroughly shaken. Then 0.5 ml urine was pipetted in a glass tube with 0.9 ml 2% glutaraldehyde in 0.134 M phosphate buffer, pH 7.4. The patients were allocated at random to double-blind treatment with pivmecillinam HCl 200 mg 3 times

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and pyuria reflect entirely different

gh correlated phenomena. The bacteriuria is the bacterial invasion into the urinary tract; pyuria reflects the inflammatory response of urinary tract.

The present observations on the relation between bacteria and pyuria are in agreement with the usual model of a primary bacterial infection and a secondary inflammatory response of engaged tissue. The significance of bacteriuria and pyuria has not been recognized by many clinicians. The interpretation of various combinations and magnitudes of bacteriuria and pyuria has nevertheless been subject to keen interest from previous workers (1, 3, 4, 9). The contribution of the present study to the time course of bacteriuria and pyuria in treated UTI has been mapped by means of permanent quantitative urine sediment.

The clinical implications of the present study are that bacteriuria without pyuria should not be treated in the absence of imperative clinical symptoms. If the patient seems not to be hurt by this bacterial invasion since there is no inflammatory response.

Treatment of a UTI may lead to a sterile flora, which will disappear spontaneously.

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daily, or the combination pivmecillinam HCl 100 mg and pivampicillin 125 mg 3 times daily, for 7 days. Urine was sampled for the PQ sediment on days 0-7, 9, 24. The glutaraldehyde-fixed urine was kept under refrigeration for 1-7 days before the preparation of the slide specimens. The slide preparations were obtained by means of a cytocentrifuge (Shandon Elliot Cytospin[®]), 1000 rpm, 10 minutes, 0.2 ml glutaraldehyde-suspended urine, double preparations from each sample. One of the twin preparations was stained according to May-Grunwald Giemsa, the other by means of hematoxylin-eosin. The cells and bacteria were counted in a Zeiss Photomicroscope at $\times 1,000$ magnification along a diameter of the preparation (52 visual fields) and converted into corpuscles per μ l urine in the original sample, as described in a previous study (5). The twin preparation with the highest cell count was taken to represent the cellularity of the urine.

RESULTS

There was a time lag between the bacteriuria and the pyuria in both the mecillinam-treated group of patients (Fig 1) and the combination-treated group of patients (Fig 2). The bacteriuria disappeared completely within 1-2 days of antimicrobial therapy.

The pyuria was reduced more slowly and approached insignificant levels, 10 cells/ μ l urine, on day 4 (Fig 2) and day 7 (Fig 1), respectively. Bacteriuria reappeared quicker than pyuria after withdrawal of antimicrobials (Figs 1, 2).

DISCUSSION

Geriatric inpatients of the present type, with varying degrees of dementia and urinary incontinence, have a tendency to acquire recurrent bacteriuria, often without signs and symptoms of UTI. The present study by means of the PQ sediment elucidates the correlation between bacteriuria and pyuria in these patients.

There was a time lag between bacteriuria and pyuria, evident both at the beginning and at the end of UTI (Figs 1, 2). Bacteriuria disappeared quicker than pyuria during antimicrobial treatment and reappeared quicker than pyuria after withdrawal of treatment.

It is reasonable to assume that the time lag between bacteriuria and pyuria in the course of antibiotic-treated UTI (Figs 1, 2) is due to the fact

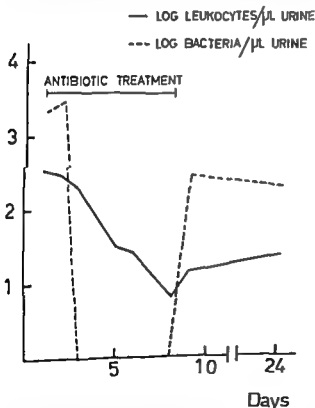


Fig 1 The time lag between bacteriuria and pyuria during mecillinam treated urinary tract infection in 15 geriatric inpatients without indwelling catheters. Median values 1 = 10 cells/ μ l urine.

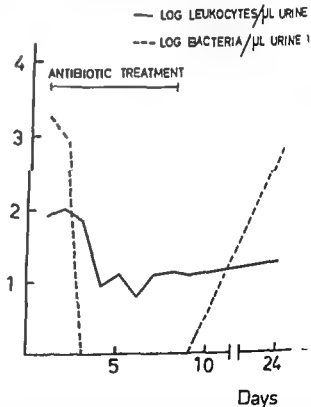


Fig 2 The time lag between bacteriuria and pyuria during urinary tract infection treated with the combination mecillinam/ampicillin in 15 geriatric inpatients without indwelling catheters. Median values 1 = 10 cell/ μ l urine.

bacteriuria and pyuria reflect entirely different
 correlated phenomena. The bacteriuria
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The present observations on the relation between
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 classical model of a primary bacterial infection and a
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 The significance of bacteriuria and pyuria has not
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 interpretation of various combinations and magni-
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 been subject to keen interest from previous workers
 (1, 3, 4, 9). The contribution of the present study
 at the time course of bacteriuria and pyuria in
 antibiotic-treated UTI has been mapped by means of
 permanent quantitative urine sediment.

The clinical implications of the present study are
 that bacteriuria without pyuria should not be
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Treatment of a UTI may lead to a sterile
 bacteria which will disappear spontaneously.

We thank Mrs Patricia Wetterberg for revising the
 manuscript. This study was supported by grant No 5362
 from the Swedish Medical Research Council to Astrid
 Wetterberg.

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daily, or the combination pivmecillinam HCl 100 mg and pivampicillin 125 mg 3 times daily, for 7 days. Urine was sampled for the PQ sediment on days 0-7, 9, 24. The glutaraldehyde-fixed urine was kept under refrigeration for 1-7 days before the preparation of the slide specimens. The slide preparations were obtained by means of a cytocentrifuge (Shandon-Elliot Cytospin®) 1000 rpm, 10 minutes, 0.2 ml glutaraldehyde-suspended urine, double preparations from each sample. One of the twin preparations was stained according to May Grunwald-Giemsa, the other by means of hematoxylin-eosin. The cells and bacteria were counted in a Zeiss Photomicroscope at $\times 1,000$ magnification along a diameter of the preparation (52 visual fields) and converted into corpuscles per μ l urine in the original sample, as described in a previous study (5). The twin preparation with the highest cell count was taken to represent the cellularity of the urine.

RESULTS

There was a time lag between the bacteriuria and the pyuria in both the mecillinam-treated group of patients (Fig 1) and the combination-treated group of patients (Fig 2). The bacteriuria disappeared completely within 1-2 days of antimicrobial therapy.

The pyuria was reduced more slowly, approached insignificant levels, 10 cells/ μ l urine, on day 4 (Fig 2) and day 7 (Fig 1), respectively. Bacteriuria reappeared quicker than pyuria after withdrawal of antimicrobials (Figs 1, 2).

DISCUSSION

Geriatric inpatients of the present type, with varying degrees of dementia and urinary incontinence, have a tendency to acquire recurrent bacteriuria, often without signs and symptoms of UTI. The present study by means of the PQ sediment elucidates the correlation between bacteriuria and pyuria in these patients.

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It is reasonable to assume that the time lag between bacteriuria and pyuria in the course of antibiotic-treated UTI (Figs 1, 2) is due to the fact

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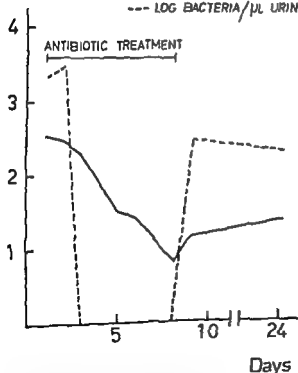


Fig 1 The time lag between bacteriuria and pyuria during mecillinam-treated urinary tract infection in 15 geriatric inpatients without indwelling catheters. Median values. 1 = 10 cells/ μ l urine.

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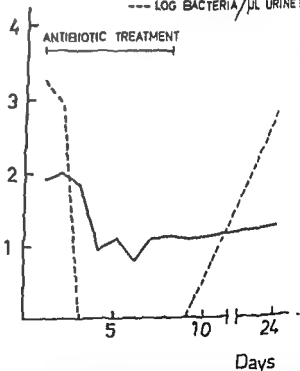


Fig 2 The time lag between bacteriuria and pyuria during urinary tract infection treated with the combination mecillinam-ampicillin in 15 geriatric inpatients without indwelling catheters. Median values. 1 = 10 cells/ μ l urine.

bacteriuria and pyuria reflect entirely different but correlated phenomena. The bacteriuria is the bacterial invasion into the urinary tract; pyuria reflects the inflammatory response of the urinary tract.

The present observations on the relation between bacteriuria and pyuria are in agreement with the usual model of a primary bacterial infection and a secondary inflammatory response of engaged tissue. The significance of bacteriuria and pyuria has not yet been recognized by many clinicians. The pretation of various combinations and magnitudes of bacteriuria and pyuria has nevertheless been subject to keen interest from previous workers (1, 3, 4, 9). The contribution of the present study at the time course of bacteriuria and pyuria in antibiotic-treated UTI has been mapped by means of permanent quantitative urine sediment.

The clinical implications of the present study are that bacteriuria without pyuria should not be regarded as the absence of imperative clinical symptoms; the patient seems not to be hurt by this bacterial invasion since there is no inflammatory response.

Treatment of a UTI may lead to a sterile pyuria which will disappear spontaneously.

I thank Mrs. Patricia Wetterberg for revising the manuscript. This study was supported by grant No. 5362 from the Swedish Medical Research Council to Astrid Berg.

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A PROTEIN ANTIGEN CHARACTERISTIC OF *BRANHAMELLA CATARRHALIS*

Serological Identification of the Genus

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Abstract. A protein antigen characteristic of *Branhamella catarrhalis*. Serological identification of the genus. Acta path. microbiol. scand. Sect. B 88: 281-286, 1980.

Reaction patterns of sonicated, acid-extracted and other extracts from *Branhamella catarrhalis* were examined by double diffusion in gel technique using antiserum to *B. catarrhalis*. Acid extract gave rise to 4 distinct precipitates. One of these lines was further studied. The bacterial component responsible for this line was trypsin-sensitive, indicating that it was a protein. It was anodally localized by crossed immunoelectrophoresis. By absorption of antiserum with whole bacteria, the precipitating capacity of the serum was diminished, suggesting that the protein antigen (P antigen) was exposed on the bacterial surface. Fragments of IgG from antiserum, but not from normal rabbit serum, precipitated the antigen, indicating that it was a true antigen-antibody reaction. It was possible to make an IgG preparation monospecific for the P antigen by absorbing antiserum with trypsinized bacterial extract. 1 strain of *B. catarrhalis*, 9 strains of *N. gonorrhoeae*, 10 strains of *N. meningitidis*, 12 other *Neisseria* spp. and 2 strains of *H. influenzae* were investigated for presence of cross-reacting surface antigens using IgG monospecific for the P antigen and ¹²⁵I-labelled protein A from *Staphylococcus aureus*. After antibody exposure, all 31 strains of *B. catarrhalis* showed abundant uptake of protein A. No significant uptake was detected on any other investigated strain. Hence, the P antigen appears to be characteristic of *B. catarrhalis*. The possibility of a serological identification of the species is introduced. Precipitating antibodies against the P antigen were demonstrated in 69% of normal human sera.

Key words: *Branhamella catarrhalis*, antigens, serological identification, protein A.

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In 1970 the former name *Neisseria catarrhalis* was transferred to a new genus *Branhamella* (3). A new generic name was proposed on the basis of comparison of DNA base content between *B. catarrhalis*, the type species of genus *Neisseria*, and 6 *Neisseria* species.

Studies on cell envelope proteins (17) and flagellar and free lipopolysaccharides (7) also indicate that there are extensive differences of cellular composition and physiology between the genus *Neisseria* and the new genus *Branhamella*.

Previous reports published during the last 20 years have commonly held

view of *B. catarrhalis* as merely being a harmless saprophyte of the upper respiratory tract. A pathogenic role of this bacterium has been indicated in acute otitis media (4, 8), maxillary sinusitis (2), chronic bronchitis and bronchopneumonia in the compromised host (14) and acute laryngitis in the adult (18). Case reports concerning bacteremia with meningitis have also been published (5).

The present report concerns a protein antigen from *B. catarrhalis* characteristic of this species. This antigen could be precipitated by a majority of sera from apparently healthy human individuals by the double diffusion in gel technique.

Bacterial Strains

Twenty-nine *B. catarrhalis* strains isolated from clinical specimens were identified as described by Berger (1) *N* spp. *N. flavescens* ATCC 13120 *N. subflava* ATCC 11076 *N. flava* ATCC 14221 *N. perflava* ATCC 10555 *N. sicca* ATCC 9913 *N. canis* 14687 *N. caviae* ATCC 14659 *N. cinerea* 194 (U Berger 1973) *N. ovis* ATCC 19575 *N. mucosa* ATCC 19598 *N. hemolyans* ATCC 10379 *N. gonorrhoeae* ATCC 11688 11689 SS 11413/40 19827/40 *N. lactamicus* ATCC 23970 and *Branhamella catarrhalis* ATCC 8193 NCTC 4103 were kindly supplied by Dr I Lind Statens Seruminstitut Copenhagen Denmark and *N* spp. *N. meningitidis* types A B C D X Y Z W 135 29E *N. gonorrhoeae* 670 wd 670 gr P9 wd P9gr KHJ F6 (W/III) *N. lactamicus* *N. sicca* *N. subflava* by Dr D Danielsson Regionsjukhuset Örebro Sweden Two *H. influenzae* strains one type b with capsule and one not typed without capsule were isolated from clinical specimens in our laboratory.

The strain listed as *N. sicca* ATCC 9913 did not correspond to the fermentative criteria described for *N. sicca*. This strain had smooth colonies and could not utilize sugars. The strain should probably be considered as a strain of *N. caviae*. This observation has been made independently by others (16).

Conditions for Cultivation

For antibody binding experiments (see later) *N. meningitidis* and *N. gonorrhoeae* were cultured on GAB medium agar plates (13) at 37 °C in an 8% CO₂ atmosphere. The bacteria were harvested using sterile cotton swabs washed and suspended in PBS (phosphate buffered saline 0.03 M phosphate 0.12 M NaCl pH 7.2 + 0.02% Na azid). The other *Neisseria* spp. and the *B. catarrhalis* strains were cultured in 5 ml Todd Hewitt broth for 18 h at 37 °C under agitation washed and suspended in PBS. *H. influenzae* strains were cultured on hematin agar plates as above.

Extraction Procedures

For extraction by sonication 300 ml of Todd Hewitt broth was inoculated with a 5 ml overnight culture of the strain to be investigated and incubated for 18 h at 37 °C on a rotating platform shaker operating at 120 rev/min. The *N. gonorrhoeae* strains were cultivated on 2 GAB medium agar plates each and the *H. influenzae* strains on each 2 hematin agar plates. These strains were harvested as described above. Cells were recovered by centrifugation for 20 min at 3000 g washed twice and suspended in 5 ml of PBS. Sonication lasted 15 min at 4 °C in a Sonifer B 12 sonicator (Branson Sonic Power Co Conn USA). The cell free supernatant obtained after centrifugation for 30 min at 3000 g was kept at -20 °C until used. Hot acid extraction (as used by Lancefield for extraction of streptococcal antigens (9)) was prepared from 10 one litre Todd Hewitt broths in baffled flasks in which the bacteria were cultured for 18 h at 37 °C on a platform shaker operating at 120 rev/min. Cells were recovered by continuous centrifugation at 5000 g

washed three times in saline (0.9% w/v NaCl aq dest) and suspended in 100 ml of saline. The pH was adjusted to 2.0 with 1 M HCl. The suspension was heated for 10 min in a boiling water bath cooled on ice and pH readjusted to 7.0 with 1 M NaOH. The cell free suspension obtained after centrifugation for 30 min at 3000 g was stored at -20 °C until used as acid extra. Hot neutral (pH 7.0) and hot alkaline (pH 10.0) extra of *B. catarrhalis* strain 235 L were prepared by procedures similar to the hot acid extraction except at the different pH when boiling. In some experiments an acid extract or an intact bacterial suspension was digested with trypsin (SIGMA Chemical Co USA) 1% (w/v) for 1 h at 37 °C. The reaction was stopped by adding soya bean trypsin inhibitor (SIGMA) 1% (w/v).

Sera and Immunoglobulin Preparations

Sera were raised against *B. catarrhalis* strain 235 L in three rabbits and against strain 236 E in one rabbit using the method described by Rotta *et al* for preparation of streptococcal antisera (15). Porcine anti rabbit serum IgG (heavy and light chains) was purchased from DAK immunoglobulins Copenhagen Denmark. Pooled human IgG batch no 70972 was purchased from KAB Stockholm Sweden. Normal human sera were collected from 90 healthy blood donors.

For preparation of (Fab)₂ fragments of rabbit serum IgG 5 ml of rabbit antiserum to *B. catarrhalis* strain 235 L was separated on a Sephadex G 200 column (2.5 × 9 cm) equilibrated with PBS (flow rate 18 ml/h fraction 4.5 ml as for the following columns). Each fraction was tested by immunodiffusion for presence of IgG. Fractions containing IgG were pooled concentrated to 1 ml using a DIAFLO pm 10 ultrafiltration membrane (AMICON Corp USA) and dialysed against 0.07 M sodium phosphate buffer pH 6.3. The sample was then passed on a DEAE column (2.2 × 17 cm Pharmacia Fine Chemicals AB Uppsala Sweden) using the same buffer. The excluded fractions containing IgG were pooled concentrated to 5 ml and dialysed against 0.1 M sodium acetate buffer pH 4.5. After determination of protein content (11) the sample was digested with pepsin (SIGMA Chemical Co USA) at a pepsin/IgG ratio of 0.150 (w/v) for 15 h at 37 °C and passed on an Aca 2 column (2.2 × 17 cm LKB Stockholm Sweden) using 0.1 M sodium acetate buffer pH 4.5. The fractions of the first peak of the elution curve were pooled and concentrated to 5 ml. Part of the preparation (0.2 ml) was absorbed with *S. aureus* Cowan I (NCTC no 8530) in order to remove any remaining Fc fragments and the absorbed preparation was tested in immunodiffusion for presence of (Fab)₂ fragments.

Fab fragments of polyclonal human IgG were kindly supplied by Dr A Grubb Malmö.

For absorption of serum with whole bacteria 1 ml of serum was mixed with 25 g (wet weight) of bacteria and incubated for 30 min at 37 °C. Cells were removed by centrifugation and the supernatant was used as absorbed antiserum.

To prepare rabbit IgG specific for the P antigen 5 ml of pooled antisera (from two rabbits) raised against *B. catarrhalis* 235 L was mixed with 10 ml of trypsinized

tract, prepared from the same strain and used for 30 min at 37 °C under mild agitation. The α_2 was removed by centrifugation for 30 min at 4 °C. The supernatant was concentrated to 5 ml using FLO ultrafiltration membrane pm 10 and used on a Sephadex G 200 column equilibrated with 0.1 M Tris, 0.1 M NaCl, pH 7.5. The fractions containing monomer IgG as determined by elution volume and tests with anti-rabbit serum were pooled and used as absorbed IgG (pool volume 45 ml, 1:10 diluted) in relation to elution volume.

Immunological Methods

Diffusion in gel was performed in 0.6% (w/v) agarose (SeaKem ME Marine Colloids Div. FMC, USA) in PBS. The plates were read after 1 and 2 days at 4 °C and stained with Coomassie Brilliant Blue G250. Immunoelectrophoresis (10) was carried out

at the anode of plate of the 1st dimension, a 3 x 45 cm strip was cut and the strip placed edge to edge with agarose containing gel (1.2 or 4% v/v of antiserum) 1 mm thick. The antigens separated in 1st dimension were forced into the antibody containing gel

Detection of Surface Antigen by the Use of Absorbed Antiserum and 125 I-labelled Protein A

Protein A was purchased from Pharmacia (Uppsala, Sweden) and labelled with 125 I as described by Oakeley & Dixon (12). Bacterial strains to be investigated were heated at 55 °C for 30 min, washed 3 times and suspended in 0.5 ml of an absorbance of approximately 0.80 at 540 nm. Bacterial suspension (0.5 ml) was mixed with 100 μ l of absorbed IgG solution (see above) diluted 1:4 and incubated for 30 min at 37 °C. Two ml of PBS containing 0.1% (v/v) Tween 20 (KEBO AB, Stockholm, Sweden) was added; the suspension submitted to centrifugation for 15 min at 3000 g; the supernatant removed and the cells re-suspended in 0.5 ml of PBS. Two μ l of 125 I-labelled protein A (1 μ g) was added followed by incubation for 30 min at 37 °C. After washing in 2 ml of PBS Tween and centrifugation, the radioactivity in the bacterial pellets was counted. The radioactivity found was expressed as a percentage of the amount added.

RESULTS

Precipitation Patterns Obtained in Immunodiffusion Using Extracts from *B. catarrhalis* and Rabbit Antiserum to *B. catarrhalis*

Sonicated extract of *B. catarrhalis* strain 235 L was tested in double diffusion in gel for presence of precipitating components using antiserum to *B. catarrhalis*.

Four distinct precipitation lines were obtained among which the one appearing closest to the extract application well was more heavily stained than the others. This line will be referred to in the following as the P line and the component responsible for the precipitate as the P-component.

Acid neutral and alkaline extracts of strain 235 L were also tested for content of precipitating components. At least three of the components including the P-component were readily extractable by all three methods as judged from experiments in which interference with complete fusion was obtained when the extracts were removed.

Fab fragments of rabbit immune serum (raised against strain 235 L) – but not of normal rabbit serum – precipitated the P-component indicating that the P line was formed as an antigen-antibody interaction.

Detection of Antibodies in Human Sera against the P Component

Commercial human IgG and a total of 90 blood donor sera were examined by immunodiffusion for presence of antibodies to acid extract of strain 235 L. Commercial human IgG gave rise to two lines, one of them fusing completely with the P line obtained with rabbit immune serum to *B. catarrhalis*. This line disappeared when equal volumes of human polyclonal Fab fragments (2.5 mg/ml) and *B. catarrhalis* extract were mixed prior to testing against the commercial human IgG. In all 69% of normal human sera precipitated the P-component. A few human sera also showed a second line which was not further studied.

Characterization of the P Component

The P line disappeared after trypsin digestion of hot acid extract while the other antigens were unaffected when tested by immunodiffusion in-gel.

Trypsin-digested and crude hot acid extracts were examined by crossed immunoelectrophoresis. Using this method it was possible to distinguish five separate precipitation lines. One line appearing only in untreated extract, was apparently the P line. This

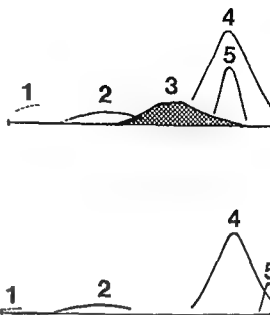
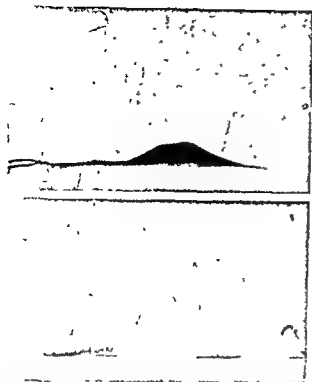


Fig 1 Crossed immunoelectrophoresis of extracts from *B. catarrhalis* strain 235 L. 4% of rabbit antiserum *B. catarrhalis* was incorporated in the gel. Anode to the right.

Top: Untreated acid extract. No. 3 represents the P-antigen. Bottom: Trypsinized extract. Note that precipitin No. 3 is absent and that the position of line No. 5 is altered towards the anode.

line was anodally localized in the crossed immunoelectrophoresis. One other precipitating component was altered by the trypsin digestion, moving more anodally after treatment of the extract. The other three precipitating components of the extract kept the same electrophoretic mobility (Fig. 1).

Rabbit antiserum to *B. catarrhalis* was absorbed with *B. catarrhalis* strain 235 L. The absorbed antiserum was unable to precipitate with the P-antigen or any of the other components precipitated by unabsorbed serum. Moreover, absorption of the serum with trypsin digested bacteria abolished all lines formed between the extract and unabsorbed serum.

After separation of the acid extract on a Sephadex G-200 column, equilibrated with PBS, the P-antigen was found in the void volume.

Investigation of *B. catarrhalis*, *Neisseria* spp. and *H. influenzae*, Using Absorbed IgG Directed against the P-Antigen and ^{125}I -Labelled Protein A

The extract-absorbed rabbit IgG was tested by immunodiffusion for the presence of antibodies against acid extract of *B. catarrhalis*. As judged by this method, it was monospecific to the P-antigen.

0.5 ml volumes of the standard suspension (see

above) strain 235 L were mixed with 100 μl absorbed IgG in various dilutions. Subsequent addition of protein A gave the following uptakes: *B. catarrhalis* undiluted IgG solution, 91% uptake; IgG solution diluted 1:2, 90%; 1:4, 59%; 1:16, 33%; 1:128, 17%. IgG solution diluted 1:4 was used in the following experiments.

To examine the effect of the quantity of bacteria on the protein-A uptake, the bacterial suspension used above was diluted in twofold steps and 0.5 ml of each dilution was mixed with 100 μl of absorbed IgG solution diluted 1:4. The results were as follows: undiluted suspension 58%, suspension diluted 1:2, 52%; 1:4, 46%; 1:8, 37%; 1:16, 28%; 1:32, 19%; 1:64, 13% and suspension diluted 1:128, 10% uptake. A standard suspension of strain 235 L mixed with rabbit preimmune serum gave an uptake of less than 4%.

The protein A uptake on antibody-exposed

longing to different types) and 12 different *Neisseria* spp. (one strain of each). Two strains of *H. influenzae* were also included (one encapsulated,

establish the presence of P antigen in all strains. No other strain of other species used in the study contained P antigen as judged by this method.

DISCUSSION

Extracts of *B. catarrhalis* were found to give several precipitation lines in immunodiffusion against rabbit antiserum to *B. catarrhalis*; one of the lines referred to as the P line was more prominent than the others. The formation of the P line between hot hydrochloric acid extract and Fab₂-fragments of IgG from rabbit immune serum (but not with such fragments of normal rabbit serum) indicated that the P line resulted from an antigen-antibody reaction. Moreover, the formation of the P line between polyclonal human IgG and the extract was inhibited by addition of Fab-fragments of polyclonal human IgG to the extract. These findings indicated that the P line was probably not the result of a reaction between *B. catarrhalis* component and antibody independent of the specificity of the antibody; such a reaction has been described between IgD and *B. catarrhalis* (6).

IgD myeloma protein did not precipitate with extracts of *B. catarrhalis* nor did it inhibit the formation of the P line when mixed with extract before immunodiffusion (unpublished observation).

The trypsin sensitivity of the bacterial component (P antigen) responsible for the P line indicates that it is a protein.

By absorption of rabbit antiserum to *B. catarrhalis* with trypsinized extract of the same strain it was possible to prepare an antiserum specific for the P antigen. When using this absorbed serum and radio-labelled protein A from *Staphylococcus aureus* the P antigen could be detected as a surface antigen on the intact bacteria. Significant antibody binding to *B. catarrhalis* was detected in amounts of bacteria down to 1% of the quantity of bacteria used in the studies designed to detect P antigen among different bacterial species. In all 31 strains of *B. catarrhalis*, 31 of *Neisseria* species and 2 *H. influenzae* strains were investigated regarding their capacity to bind anti-P antibodies. The results revealed an abundant uptake in all 31 strains of *B. catarrhalis* but no significant uptake in other species, indicating that the P antigen is a surface structure characteristic of *B. catarrhalis*.

The variable results 13–38% binding of the different *B. catarrhalis* strains were not further studied in this investigation. Among other explanations this might simply reflect differences in the concentrations of the standard suspensions.

In studies concerning the taxon relatedness of *B.*



Binding of ¹²⁵I-labelled protein A to different *B. catarrhalis* strains after addition of absorbed IgG antibodies to the mixture. Each mark represents the mean value of results obtained with one strain. A - preimmune serum and *B. catarrhalis* strain 135 L. B - *B. catarrhalis* strains II - *N. meningitidis* strains. C - *N. gonorrhoeae* strains. D - apathogenic strains. E - *H. influenzae* strains.

and one not typed without capsule). The results are shown in Fig. 2. All *B. catarrhalis* strains all showed an uptake in 13% (mean 20.5% SD 5.7%) while the other species examined all gave less than 5% (mean SD 0.7%) binding of protein A. The latter was not higher than that obtained with *B. catarrhalis* and preimmune serum (mean 3.5%). Extract of 10 strains of *B. catarrhalis* was examined for presence of P antigen by double immunodiffusion technique using acid extract of 1235 L as a reference and rabbit antiserum to *B. catarrhalis*. By this method it was possible to

catarrhalis, the genus *Neisseria* and other genera, more work is needed to elucidate the antigenic relationship. Studies on extracellular antigens have not revealed any cross-reactions between *B. catarrhalis* and *Neisseria* species (16). To my knowledge, no protein antigen with characteristics similar to the P-antigen has yet been identified.

In the present investigation, a total number of 90 human sera were tested by immunodiffusion for the ability to precipitate hot acid extracts of *B. catarrhalis*. 69% of the sera formed precipitates which fused completely with the line formed between the P-antigen and rabbit antiserum to *B. catarrhalis*. Precipitating antibodies against not further characterized structures in *B. catarrhalis* have been shown by others to be common in both normal human sera and sera from individuals recently infected with *B. catarrhalis* (2). A pathogenic role of *B. catarrhalis* has been indicated in several studies (see above). Studies are in progress concerning the role of the P-antigen in the virulence of the bacteria, and the antibody response during infection.

Identification of *B. catarrhalis* has hitherto relied on biochemical tests: colony morphology, and growth characteristics. *B. catarrhalis* is relatively inactive biochemically, and the oxidase and catalase production as well as nitrate reduction are characteristics shared with several other closely related microorganisms. The fermentation tests for recognition of the bacteria are generally based on negative reactions. A serological identification of this genus — by means of identification of the P-antigen — would seem to be desirable. A quick and simple method for the serological identification of *B. catarrhalis* on the basis of the P-antigen is under development.

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STUDIES ON THE PATHOGENICITY OF *YERSINIA ENTEROCOLITICA* AND *Y. ENTEROCOLITICA*-LIKE BACTERIA

Enterotoxin Production at 22 °C and 37 °C by Environmental and Human Isolates from Scandinavia

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perud G Studies on the pathogenicity of *Yersinia enterocolitica* and *Y. enterocolitica* like bacteria enterotoxin production at 22 °C and 37 °C by environmental and human isolates from Scandinavia
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gether 412 strains of *Yersinia enterocolitica* and *Y. enterocolitica* like bacteria from environmental human sources were examined for their ability to produce enterotoxin at 22 °C and 37 °C using infant mouse assay. A total of 73 strains were positive at 22 °C only. Another 28 strains were positive both at 22 °C and 37 °C. Of these 28 strains, 26 were sucrose and aesculin non fermenting (all were Voges Proskauer negative). All of these strains were isolated from non human sources. All 13 strains belonging to O serogroup 28 produced enterotoxin at 37 °C. The results indicate that enterotoxin production at 22 °C is widespread in *Y. enterocolitica* with the highest prevalence among human clinical isolates. Enterotoxin production at both 22 °C and 37 °C seems to be common in *Y. enterocolitica* (sucrose non fermentors). Enterotoxin production is apparently rare in *Y. frederiksenii* (sucrose fermentors) and in *Y. intermedia* (lactose and melibiose fermentors).

Y words *Yersinia enterocolitica* enterotoxin

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18 strains of *Yersinia enterocolitica* produce a stable enterotoxin *in vitro* which is active in the mouse and rabbit ileal loop assay (2, 5, 11, 13). A close similarity between the *Y. enterocolitica* enterotoxin and the heat stable (ST) toxin of *Escherichia coli* has been shown (2). Robins Browne *et al.* (13) stated that, in order to sharing a number of physicochemical features these two enterotoxins are similar in mechanism of action. Pat *et al.* (12) studied human and non human isolates from Canada and stated that enterotoxin production (at room temperature) in *Y. enterocolitica* with

the highest prevalence among strains associated with human infection. However, none of the authors referred in above report enterotoxin production at the human body temperature. Likewise, documentation of enterotoxin production *in vivo* and under anaerobic conditions is lacking. Thus, a clinical significance of the *Y. enterocolitica* enterotoxin has not been established.

In a current communication from this laboratory it was reported that some strains of *Y. enterocolitica* O-serogroup 28 produced enterotoxin at 37 °C as well as at 22 °C (9). The purpose of the present study was to survey the prevalence of enterotoxin production at 37 °C and 22 °C among

Y. enterocolitica and *Y. enterocolitica*-like bacteria isolated from environmental and human sources in Scandinavia

Preparation of Sterile Culture Filtrates

Sterile culture filtrates were obtained from *br* cultures incubated on a roller drum (60 rev/min) room temperature (approximately 22 °C) and at 37 for 48 hours (10)

MATERIALS AND METHODS

Bacterial Strains

A total of 412 strains of *Y. enterocolitica* and *Y. enterocolitica*-like bacteria were examined. All strains were stored as stab cultures at 4 °C. Three groups of strains may be discerned (Table 1).

Environmental strains. A total of 331 strains from environmental sources were examined. Included are 330 strains isolated from the intestinal contents of apparently healthy, wild-living mammals, birds and fish and from water samples and soil in Scandinavia. Most of these strains have been described in previous communications (6, 7, 8). Also included is one strain from a diseased goat (O-serogroup 2).

Human strains. Included are 48 strains from human stools. Twenty of these strains were isolated from healthy humans (Kapperud, *G.*, manuscript). The remaining 28 strains were isolated at the National Institute of Public Health, Oslo, (courtesy of Dr J. Lassen) from patients with gastroenteritis. Twenty-two of these strains belonged to O-serogroup 3/biotype 4 (Wauters' biotype scheme).

Reference strains. A total of 33 type strains representing *Y. enterocolitica* O-serogroup 1-34 were examined. The strains were received from Institut Pasteur, Paris.

Enterotoxin Assay

The culture filtrates were tested for enterotoxin activity using the infant mouse assay (4) as previously described (9).

RESULTS

Of 412 isolates of *Y. enterocolitica* and *Y. enterocolitica*-like bacteria, 101 (25%) produced *E. coli* like enterotoxin at 37 °C and/or 22 °C as indicated by the infant mouse assay. Of these 101 strains, 1 were positive at 22 °C, only. The remaining strains were positive both at 22 °C and 37 °C (Table 1). Among the strains examined, the prevalence of enterotoxigenicity varied with the source of isolation, O-serogroup and biochemical properties of the strains.

Source of Isolation

Enterotoxin production was indicated for 1 (19%) of 331 environmental strains, 29 (60%) 48 human strains and for 8 (24%) of 33 reference strains (Table 1). The highest prevalence of enterotoxigenicity was observed among the strains isolated

TABLE 1 Relationship between Enterotoxin Production and Source of Isolation of *Y. enterocolitica* and *Y. enterocolitica*-Like Bacteria

Source	No. of strains	No. positive at		Total (%) ^a
		22 °C	22 °C and 37 °C	
<i>Environmental strains</i>				
Shrews	35	4	14	51
Small rodents	96	26	5	32
Water	57	4	6	18
Fish	129	1	1	2
Birds	7	3	—	—
Foxes	5	—	—	—
Soil	1	—	—	—
Goat	1	—	—	—
<i>Human strains</i>				
Gastroenteritis	28	20	—	71
Healthy	20	9	—	45
<i>Reference strains</i>	33	6	2	24
<i>Total</i>	412	73	28	25

TABLE 2 Relationship between Enterotoxin Production and O Serogroups of *Y. enterocolitica* and *Y. enterocolitica* Like Bacteria

Serogroup	Environmental strains			Human strains		
	Nil tested	No positive at		No tested	No positive at	
		22 °C	22 °C and 37 °C		22 °C	22 °C and 37 °C
1	9	7	1	—	—	—
3	7	1	—	22	20	—
4	35	4	—	2	1	—
5	2	—	—	3	2	—
6	41	9	—	6	2	—
7	5	1	—	1	1	—
11	8	—	4	—	—	—
12	11	3	—	—	—	—
13	5	—	—	4	1	—
16	13	4	—	2	1	—
28	12	—	12	—	—	—
NA(G)	130	9	9	7	1	—
Other	53	—	—	1	—	—
Total	331	38	26	48	29	—

Non agglutinable

from human patients with gastroenteritis followed by the strains from healthy wild living mammals, deer and fish (in order of decreasing prevalence) and the 18 strains which produced enterotoxin at 7 °C were isolated from non human sources of terrestrial and aquatic ecosystems. Altogether 19 (51%) of these strains were isolated from healthy wild living small mammals. Shrews (Soricidae) produced 14 (50%) of the strains which produced enterotoxin at 37 °C. Forty per cent of the strains recovered from shrews showed this property.

O-serogroups

Among the environmental isolates enterotoxin production was indicated for strains belonging to the different O-serogroups and for non agglutinable strains (Table 2). Positive test was obtained in 22% of O-serogroup 11 and 11% of O-serogroup 4, two of the most frequently encountered O-serogroups in Scandinavian ecosystems among the reference strains enterotoxin production was indicated for O-serogroup 5, 5-27, 12-25, 14, 20, 22 and 28. Further enterotoxin production was indicated for 20 (91%) of 22 strains belonging to O-serogroup 3/biotype 4 isolated from human patients with gastroenteritis. However none of these strains were enterotoxigenic at the human body temperature. Enterotoxin production at 37 °C was indicated for environmental strains belonging

to O-serogroup 1, 11, 28 and non agglutinable. In addition two of the reference strains showed this

Biochemical Properties

Among the environmental isolates the highest prevalence of enterotoxigenicity was observed with

37 °C were sucrose and aesculin non fermenting and all were Voges Proskauer negative (at 22 °C and 37 °C). A very low prevalence of enterotoxigenicity was detected among the rhamnose fermenting strains and among the rhamnose and melibiose fermenting strains.

DISCUSSION

The results indicate that enterotoxin production is widespread among *Y. enterocolitica* and *Y. enterocolitica* like bacteria from Scandinavia. The highest prevalence of enterotoxigenicity was observed among strains associated with human gastroenteritis. However none of these clinical isolates produced enterotoxin at the human body temperature.

TABLE 3 Relationship between Enterotoxin Production and Biochemical Groups of *Y. enterocolitica* and *Y. enterocolitica*-like Bacteria Isolated from Environmental Sources

Biochemical group ^a	No. of strains	No. positive at		Total (%)
		22 °C	22 °C and 37 °C	
<i>Y. enterocolitica</i> (YE)	136	26	2	19
<i>Y. enterocolitica</i> -like bacteria				
Sucrose - (S)	49	9	24	53
Rhamnose + (R)	55	1	-	2
Rhamnose + melibiose + (RM)	21	1	-	5
Other ^b	70	1	-	1

^a Definition of biochemical groups

YE	rhamnose -	sucrose +	melibiose -	
S	» -	» -	» -	
R	» +	» +	» -	
RM	» +	» +	» +	

All groups were positive for cellobiose, sorbose and ornithine decarboxylase

+ = fermenting.

- = non-fermenting

^b Other combinations of the characters listed above

Further, both humans and animals were healthy carriers of enterotoxigenic strains. Thus, the clinical significance of the *Y. enterocolitica* enterotoxin remains uncertain.

Enterotoxin production at 37 °C was indicated for a group of sucrose and aesculin non-fermenting, Voges-Proskauer negative strains most of which were isolated from the intestinal contents of apparently healthy, wild-living small mammals. Similar strains have occasionally been isolated from atypical clinical entities in humans (1). With this exception, no clinical role has so far been ascribed to such strains. The present data, however, suggest that the pathogenicity of these strains should be reconsidered.

Pal et al. (12) reported that 64.7% of 414 *Y. enterocolitica* strains from Canada were positive in the infant mouse assay after incubation at room temperature. This relatively high percentage compared to the present results may be due to the dominance of human clinical isolates in their work. Further, Pal et al. (12) reported that the prevalence of enterotoxin production was 50% among strains isolated from animals. They did not give any information regarding the kinds of species studied or the clinical significance of these isolates. However, the reported prevalence is not significantly different from the present observation on healthy, wild-living small mammals (Table 1).

Brenner et al. (3) examined *Y. enterocolitica* and *Y. enterocolitica*-like bacteria by DNA-hybridization. They proposed four species corresponding to distinct DNA-relatedness groups all of which can be biochemically defined: *Y. kristensenii* (sucrose non-fermentors), *Y. frederiksenii* (rhamnose fermentors), *Y. intermedia* (rhamnose and melibiose fermentors) and *Y. enterocolitica* (typical isolates). The present results indicate that these species have different enterotoxigenic properties (Table 3). Enterotoxin production both at 37 °C and 22 °C seems to be common in *Y. kristensenii*. Enterotoxin production at 22 °C, only, is widespread in *Y. enterocolitica*, with the highest prevalence among human clinical isolates. Enterotoxin production is apparently rare in *Y. frederiksenii* and *Y. intermedia*.

Among the strains studied *Y. kristensenii* dominated in small mammals, especially shrews, whereas *Y. frederiksenii* prevailed in fish (Kapperud, manuscript). Water contained both species in approximately equal proportion. This condition may account for the differences in the prevalence of enterotoxin production observed between strains from small mammals, fish and water (Table 1).

A clinical significance of the *Y. enterocolitica* enterotoxin could not be established in this work. Recently, however, it was shown that some strains of *Y. enterocolitica* and *Y. enterocolitica*-like bac-

produced enterotoxin in refrigeration temperature which is a common storage condition for foods. A possible role of this enterotoxin in food poisoning has been proposed (2, 10). The omnipresence of enterotoxigenic strains of *Y. enterocolitica* and *Y. enterocolitica* like bacteria in nature, as recently indicated, suggests that this topic deserves further attention.

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STUDIES ON THE PATHOGENICITY OF *YERSINIA ENTEROCOLITICA* AND *Y. ENTEROCOLITICA*-LIKE BACTERIA

2. Interaction with HeLa Cells among Environmental and Human Isolates from Scandinavia

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Kapperud G. Studies on the pathogenicity of *Yersinia enterocolitica* and *Y. enterocolitica* like bacteria. 2. Interaction with HeLa cells among environmental and human isolates from Scandinavia. Acta path microbiol scand Sect B 88: 293-297 1980.

A total of 412 strains of *Yersinia enterocolitica* and *Y. enterocolitica* like bacteria were examined for their ability to interact with HeLa cell monolayers. Of 331 isolates from environmental sources in Scandinavia only three strains biochemically classified as *Y. pseudotuberculosis* were invasive for HeLa cells. Invasiveness was also indicated for one strain (O serogroup 2) from a diseased goat. Another eight strains adhered firmly to the cell surface in great numbers. All of 22 strains belonging to O serogroup 3 from human patients with gastroenteritis were invasive. Seven strains of O serogroup 3 from small rodents and water were non-invasive. Among 33 reference strains representing *Y. enterocolitica* O serogroup 1-34 invasiveness was indicated for strains with known pathogenicity (O serogroup 1, 2, 3, 5-27, 8, 9). However, some strains belonging to O-serogroups with uncertain clinical significance were also invasive for HeLa cells. A close correlation between invasiveness and enterotoxin production was demonstrated for the 22 human clinical isolates belonging to O serogroup 3.

Key words: *Yersinia enterocolitica*, invasiveness, HeLa cells.

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The ability of certain strains of *Yersinia enterocolitica* to penetrate HeLa cells has been reported by several authors (6, 8, 9, 10). This property seems to be closely related to the pathogenicity of *Y. enterocolitica* since almost exclusively strains with recognized clinical significance were invasive for HeLa cells. Une (9) concluded that this model reflected well the ability to invade epithelial linings in vivo. Therefore invasion of HeLa cells might serve as an indicator of the pathogenicity of *Y. enterocolitica*.

In the present study, HeLa cell invasiveness was investigated in a strain material previously examined for the ability to produce enterotoxin (5). Enterotoxin production is widespread among *Y. enterocolitica* and *Y. enterocolitica*-like bacteria from Scandinavia. The highest prevalence of enterotoxigenicity was observed among human clinical isolates. However, enterotoxin production was also indicated for a broad spectrum of strains with uncertain clinical significance.

The aims of the present investigation were 1) to survey the ability to penetrate HeLa cells among *Y.*

enterocolitica and *Y. enterocolitica* like bacteria from environmental and human sources in Scandinavia 11) to ascertain if any correlation is portrayed between HeLa cell invasiveness and enterotoxin production

MATERIALS AND METHODS

Bacterial Strains

A total of 412 strains of *Y. enterocolitica* and *Y. enterocolitica* like bacteria and seven reference strains of *Y. pseudotuberculosis* were examined. The strains have been described in a concurrent article (5). Three groups of strains may be discerned:

Environmental strains 331 isolates from varied ecological and geographical sources in Scandinavia (Table 1)

Human strains 48 strains from healthy and diseased humans in Norway (Table 2)

Reference strains 33 type strains of *Y. enterocolitica* and seven type strains of *Y. pseudotuberculosis* (Table 3)

Preparation of Bacterial Inoculum

The strains were grown in trypticase soy broth (BBL 11767) with 0.6% yeast extract (Difco 0127). The broths were incubated at 22 °C for 18–20 hours. Prior to challenge of the HeLa cells the cultures were centrifuged and the bacterial pellets were re-suspended in identical volumes of Eagle's basal medium (BME) with sodium bicarbonate (1g per ml) and 5% foetal bovine serum (FBS).

Cultivation of HeLa Cells

supplied with 1 ml cell suspension in BME containing bicarbonate and 10% FBS. Streptomycin (100 iu per ml) and vancomycin (100 µg per ml) were added to the medium. The cell inoculum was adjusted to approximately 8×10^4 cells per well to achieve a cell layer with blank areas (leopard spots) to increase the surface available for bacterial penetration. The cells were incubated overnight. All incubations were performed at 37 °C in 5% CO₂ atmosphere with saturated humidity.

Challenge of Cell Cultures

The cell monolayers were washed three times with temperate (37 °C) Dulbecco's complete phosphate buffered saline (PBS) (pH 7.2) to reduce the presence of streptomycin/vancomycin and re-supplied with fresh BME (5% FBS) without antibiotics. After incubation for one hour the cells were washed with PBS (37 °C) to minimize the concentration of antibiotics. Finally the cells were incubated with 1 ml fresh BME (5% FBS without antibiotics) and inoculated with 0.025 ml of a bacterial suspension containing approximately 3×10^{12} living bacteria per ml.

Following an incubation for 90 minutes the medium was decanted and the cells were washed three times with PBS (37 °C) to remove extracellular bacteria. Fresh BME

(5% FBS) with streptomycin/vancomycin was added. The streptomycin was used to inhibit the growth of remaining extracellular bacteria and thereby protect cells from re-infection. The cultures were incubated three hours.

Staining and Microscopy

After incubation the medium was decanted and cells were washed three times with PBS. The HeLa cells were fixed with absolute methanol for one minute. Coverslips were removed, mounted on slides and stained with 4% Giemsa solution for 30 minutes. T Preparates were examined by oil immersion microscope at a magnification of 1000×

Interpretation of the Results

The strains were considered invasive if a majority of the HeLa cells contained more than 10 intracellular bacteria. The number of intracellular bacteria varied considerably between individual strains. However, further quantification was attempted. It was somewhat difficult to distinguish intracellular (invasive) from extracellular (adhesive) bacteria. In practice invasiveness was evaluated by visual comparison with strain IP 11 (Institut Pasteur Paris). Lee *et al.* (6) confirmed invasiveness of this strain by electron microscopy. Strains showing ambiguous results were retested 3-times.

RESULTS

Environmental Strains

Twelve of 331 strains of *Y. enterocolitica* and *Y. enterocolitica* like bacteria from environmental sources in Scandinavia interacted with HeLa cells (Table 1).

Only four (1%) strains were invasive. One of these strains (O serogroup 2) was isolated from diseased goat. Invasiveness was also indicated for three strains biochemically classified as *Y. pseudotuberculosis* isolated from apparently healthy wild living small rodents in Denmark (4). Each of these isolates were agglutinated by *Y. enterocolitica* O antiserum 4–33 and 21 and by *Y. pseudotuberculosis* O antiserum IIB.

A total of eight strains adhered firmly to the cell surface and to the glass. Seven of these strains belonged to O serogroup 4/biotype 1 (Wauters biotype scheme) isolated from small rodents. It was not possible to determine the invasiveness of these strains owing to the great number of bacteria attached extracellularly. The phenomenon was reproducible on several repeated examinations. Additional washing with PBS was not sufficient to remove the bacteria.

Human Strains

All of 22 strains belonging to O serogroup 3/biotype 4 from human patients with gastroenteritis

TABLE 1 Interaction with HeLa Cells among *Y. enterocolitica* and *Y. enterocolitica* Like Bacteria Isolated from Environmental Sources in Scandinavia

Serogroup ^a	No. of strains tested	No. invasive	No. adhesive
	9	—	—
	1	1	—
	7	—	—
	33	—	7
	2	—	—
	41	—	—
	5	—	—
	8	—	—
	11	—	—
	5	—	—
	10	—	—
	1	—	—
	13	—	—
	9	—	—
	12	—	—
	23	3	—
	130	—	1
	5	—	—
	4	—	—
Total	331	4	8

^a The figures indicate antigenical relationship to O groups of *Y. enterocolitica*. Roman figures O groups of *Y. pseudotuberculosis*.
^b Used (agglutinated by 2-5 of the antisera 4 16 18 11 1A 1B and 11B in ten different combinations).
^c Not agglutinable.

were invasive (Table 2). There was a tendency for these strains to adhere extracellularly. Detachment of HeLa cells from the coverslips was also observed. In contrast, seven strains of O serogroup 3 isolated from small mammals and water, were non invasive (Table 1). None of these strains could be ascribed to biotype 4.

Another six isolates from human patients, belonging to other O serogroups, did not interact with the HeLa cells. However, the clinical significance of these strains has not been confirmed. Further, neither invasion nor adhesion was discovered among 20 strains isolated from healthy humans.

Reference Strains

A total of 15 (45%) of 33 reference strains representing *Y. enterocolitica* O-serogroup 1-34 were invasive (Table 3). Invasiveness was also indicated for six of the seven reference strains of *Y. pseudotuberculosis*.

Relationship between HeLa Cell Interaction and Enterotoxin Production

A close correlation between invasiveness and enterotoxigenicity was demonstrated for human clinical isolates belonging to O-serogroup 3/biotype 4. All 22 isolates were invasive, and 20 of these also produced enterotoxin at 22 °C.

Interaction with HeLa cells was not shown for any of the 64 environmental strains which were enterotoxigenic.

Three reference strains were both invasive and enterotoxigenic (O-serogroup 5-27 20 and 22).

TABLE 2 Interaction with HeLa Cells among *Y. enterocolitica* and *Y. enterocolitica* Like Bacteria Isolated from Healthy and Diseased Humans in Norway

Serogroup	Healthy humans		Diseased humans	
	No. tested	No. invasive	No. tested	No. invasive
	—	—	22	22
	1	—	1	—
	2	—	1	—
	5	—	3	—
	1	—	—	—
	4	—	—	—
	—	—	—	—
	1	—	1	—
	6	—	1	—
	—	—	1	—
Total	20	—	28	22

^a Not agglutinable.

TABLE 3 *HeLa Cell Invasiveness and Enterotoxigenicity among Reference Strains of Y enterocolitica and Y pseudotuberculosis*

O serogroup	Strain ^a	Source	Invasive ^b	Enterotoxin
<i>Y enterocolitica</i>				
1	IP 6	Chinchilla	+	-
2	IP 8	Hare	+	-
3	IP 85	Swine	(+)	-
4-32	IP 96	Chinchilla	+	-
4-33	IP 1476	Water	-	-
5	IP 123	Cow	-	+
5-27	IP 47	Monkey	+	-
5-27	IP 885	Dog	+	+
6-30	IP 102	Human	-	-
6-31	IP 1477	Water	-	-
7-8	IP 106	Guinea pig	-	-
8	IP 107	Human	+	-
8	IP 311	Human	+	-
8-19	Fy 50	?	-	-
8-19	IP 842	Human	-	-
9	IP 201	Human	+	-
10	IP 551	Human	(+)	-
11-23	IP 105	Human	-	-
11-24	IP 841	Human	-	-
12-25	IP 490	Hare	-	++
12-26	IP 103	Sheep	-	+
13-7	IP 553	Human	-	-
14	IP 480	Human	-	+
15	IP 614	Human	+	-
16	IP 1475	Water	-	-
16-29	IP 867	Human	-	-
17	IP 955	Water	-	-
18	IP 846	Human	(+)	-
20	IP 845	Human	+	+
21	IP 1110	Human	+	-
22	IP 1367	Human	+	+
28	IP 1474	Water	-	++
34	IP 1501	Human	-	-
<i>Y pseudotuberculosis</i>				
IA	402	?	+	-
IB	403	?	+	-
IIA	407	?	-	-
IIB	401	?	+	-
III	405	?	+	-
IV	404	?	+	-
V	406	?	+	-

^a Strains with the IP prefix were received from Institut Pasteur, Paris. The other reference strains were obtained from Dr. Winblad, Malmö.

^b + = positive reaction - = negative reaction (+) = weak reaction + + = enterotoxin production both at 22 and 37 °C

DISCUSSION

Most of *Y enterocolitica* and *Y enterocolitica* like bacteria from environmental and human sources in Scandinavia were non-invasive in the HeLa cell model employed in this study. Invasiveness was

indicated for all strains otherwise recognized pathogenic and furthermore for three strains related to *Y pseudotuberculosis*. HeLa cell invasiveness is a documented feature of *Y pseudotuberculosis*. These results are in accordance with the

ed from North America by Lee *et al* (6) and Japan by Une *et al* (10)

et al (6) suggested that strains that cannot penetrate HeLa cells which have a very weak resistance against bacterial infection are most probable to invade epithelial cells *in vivo*. On the other hand invasiveness may be more prevalent in *Y. enterocolitica* and *Y. enterocolitica* like strains than indicated by laboratory models. Invasiveness in *Y. enterocolitica* is mediated by a plasmid (3, 11). Such a factor may be lost after cloning of the strains. Further 131 of the strains were isolated from wild living mammals which were trapped by methods to select active individuals and thereby reduce the ability of discovering diseased animals. This may give a false impression of the prevalence of the strains.

La cell invasiveness was demonstrated for those strains with known pathogenicity for man (O-serogroup 1, 2, 3, 5-27, 8, 9). On the other hand, some strains belonging to O serogroups of uncertain clinical significance were also found (O serogroup 4-32, 10, 15, 18, 20, 21). Similar observations were reported by Pedersen (8). However these reference strains have been used to repeated subculturing and prolonged growth which may have influenced both their invasiveness and enterotoxigenicity. The ability to consider invasion of HeLa cells could be a useful but not absolute criterion of the pathogenicity of *Y. enterocolitica* isolates. Une (9) stated that HeLa cell infectivity is not sufficient to evaluate the pathogenicity of these bacteria. This is emphasized that primarily the ability to penetrate epithelial linings and secondarily the ability to survive or multiply within macrophage cells seems to be required to establish infection with *Y. enterocolitica in vivo*. Further some kind of adhesion may be necessary to initiate invasion. The adhesion phenomenon may represent the initial stage in the invasion process. Pathogenicity in *Y. enterocolitica* and *Y. enterocolitica* like bacteria may also be ascribed to enterotoxin production (5). However adhesion, colonization or receptor binding may be needed to achieve clinical effect of *Y. enterocolitica* enterotoxin analogous to the invasion factors required by enterotoxigenic *Escherichia coli* (2).

Both invasiveness and enterotoxin production were found with very high frequency among human clinical isolates of *Y. enterocolitica* O-serogroup 3. Invasiveness study of Une *et al* (10) and the study on enterotoxigenicity of Pai *et al* (7) indicate

that a corresponding relationship likewise exists for human clinical isolates of O-serogroup 5, 8 and 9. Possibly these two pathogenicity factors act in concert to enhance the ability of *Y. enterocolitica* to provoke enteric disease.

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QUANTITATION OF M ANTIGEN IN LANCEFIELD EXTRACTS OF GROUP A STREPTOCOCCI TYPE 12 USING ELECTRO-IMMUNO ASSAY

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Eliasson, I., Svensson, M.L., Ramstorp, G., Schalen, C. & Christensen, P. Quantitation of M antigen in Lancefield extracts of group A streptococci type 12 using electro-immuno assay. *Acta path. microbiol. scand. Sect. B* 88: 299-302, 1980.

Anti type 12 serum incorporated in agarose polyethylene glycol gel in a concentration of 1.5% (vol/vol) was found to enable a distinct "rocket" precipitate in electro-immuno assay using hot hydrochloric acid extract of type 12 group A streptococci. This precipitate was removed by trypsin treatment of the extract and on addition of anti M12 typing serum but not of five other typing sera to the extract. The streptococcal component responsible for this precipitate was eluted from a CM-cellulose ion exchange column at pH 6.5. These findings demonstrated that the precipitate was caused by the M12 antigen. Crossed immuno-electrophoresis of hot hydrochloric acid extracts of three different type 12 group A streptococci showed that the electrophoretic mobility of the M12 antigens was similar in the three extracts. A linear correlation was obtained between the concentration of the M12 antigen and the height of the precipitate obtained in the electro-immuno assay using different dilutions of a standard type 12 extract. M12 antigen could thus be quantitated by the electro-immuno assay. In quantitation experiments uniformly prepared extracts of five randomly selected freshly isolated type 12 strains were found to contain from 130 to 1850% of M12 antigen respectively (expressed in % of the content of the standard type 12 extract).

Key words: Streptococci, Lancefield extract, M protein, quantitation, electro-immuno assay.

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The M antigens are important virulence factors in group A streptococci (6, 9, 14). However, few methods have been devised for quantitation of M antigen in streptococcal preparations. Becker *et al.* used a Mancini technique for quantitation of M antigen in Lancefield extracts. To our knowledge, all other immuno-chemical procedures hitherto described are modifications of the capillary precipitation method originally used for typing of group A streptococci (8). These modifications comprise estimation of the precipitate obtained between a Lancefield extract and the corresponding type-specific rabbit serum from + to ++++ (14) or measurement of the precipitate as in the Oudin technique (4, 12).

The present paper concerns quantitation of M antigen in Lancefield extracts of type 12 group A streptococci using electro-immunoassay. Measurement of M antigen in the bacteria with this method or any of the procedures described above involves cultivation, extraction and quantitation of the antigen present in the extract. Variation in quantitative results may originate from all these three steps. The aim of this investigation was primarily to describe a simple and exact method for quantitation of the M antigen content in Lancefield extracts; however, the possibility to quantitate the M12 antigen per weight unit of bacteria was also considered.

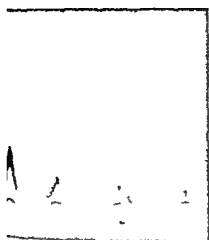


Fig. 1 Electro-immuno assay of a standard extract of type 12 group A streptococcus.

precipitate was caused by the M12 antigen. Addition of crude IgG Fc receptor from type 15 group A streptococcus (3) in equal volumes to the standard did not affect the expected height of the precipitate.

To examine the accuracy of the electro-immuno-assay system designed as above for the quantitation of M protein in *Lancefield* extracts the undiluted extract was applied in 19 wells on each of 10 different plates run on different occasions. The variation was 2.9–3.4% (SD) and the variation of the mean heights from plate to plate was 1.2% (SD). The standard diluted in 10 steps from 1:2 to 1:32 gave equally reproducible results.

Quantitation of Electrophoretic Mobility of M antigen in *Lancefield* Extracts of Three Different Type 12 Group A Strains

Extracts of three strains 1800, 300 and 305 were tested in crossed immuno-electrophoresis. Each extract gave a precipitate situated anodally in the application well. The electrophoretic mobility of the component responsible for the major precipitate was similar in all three extracts. These precipitates did not form after addition of Ca-serum to equal volumes to the extracts.

Quantitation of M protein in *Lancefield* Extracts from Type 12 Group A Streptococci Isolated from Clinical Specimens

Hydrochloric acid extracts of five freshly isolated type 12 group A streptococci were tested in electro-immuno assay. A minor precipitate was obtained that given by the trypsin resistant

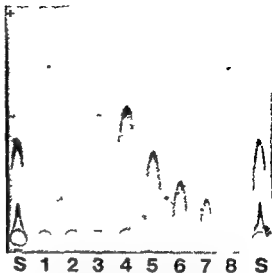


Fig. 2 Quantitation of the M protein content in a *Lancefield* extract of a freshly isolated type 12 group A streptococcus using electro-immuno assay. S: Undiluted standard extract; 1–8: Two-fold dilutions up to 1:128 of the extract to be examined. The M protein content of the extract was calculated to 1430% of the standard extract content.

component in the standard was not obtained with any of these strains. It may be noted that the freshly isolated strains were not treated with enzyme inhibitors prior to extraction, in contrast to the standard (see above). For quantitation of M12 antigen each precipitating extract was diluted to give a 'rocket' of a height between that obtained with undiluted and that obtained with 1:4 diluted standard extract (Fig. 2). The heights of the precipitates obtained with the different dilutions of the standard extract were plotted against the standard extract concentrations in a diagram after which the M12 antigen content at a suitable dilution of the extract under test could be calculated. The M12 antigen contents of the five extracts were 130, 740, 1430, 1600 and 1850% respectively of the standard extract content.

Extracts were made concomitantly of two different strains, each cultured in five bottles of 300 ml Todd Hewitt broth (from the same batch of broth). The M protein content in the extracts from one strain varied between 66 and 100% (mean 85%) and in the extracts of the other strain between 104 and 200% (mean 138%) of the standard extract content.

DISCUSSION

The M antigen in *Lancefield* extracts of type 12 group A streptococci gave distinct 'rocket' precipi-

MATERIALS AND METHODS

Bacterial Strains and Extracts

Group A streptococci type 12 were kindly supplied by Dr Burova Leningrad (strain 1800) and Dr Sramek, Prague (strains 300 and 305). Five freshly-isolated group A streptococcal strains belonging to M-type 12 were randomly selected from among the strains sent to our department. Hot hydrochloric acid extracts (8) were prepared from 18-hour cultures at 37 °C on 300 ml Todd Hewitt broth. After culturing, the streptococci were washed three times in distilled water and lyophilized. 0.10 g of the powder was then suspended in 0.15 M saline and the pH was adjusted to 2.0 with 0.1 M HCl. After heating in a boiling water bath for 10 min, the pH was neutralized to 7.0 using 0.1 M NaOH and the suspension centrifuged at 3000 g for 30 min. The volume of each extract was then adjusted to 3.0 ml with saline.

A hot hydrochloric acid extract of strain 1800 prepared from a 20-litre Todd Hewitt broth culture (13), with the addition of benzamidinium chloride and iodoacetic acid to 10 mM during washings and extraction procedures, was used as a standard preparation throughout the study and stored at -20 °C until use.

Rabbit Sera

Two different anti-type 12 sera, Lu and Ca, were used. The latter serum and anti-type 4, 28, 48 and 60 sera were M-typing sera, kindly supplied by Dr El Kholy Cairo. Unless otherwise indicated, the unabsorbed Lu serum was used as anti-type 12 serum.

Electro-immuno Assay

Electro-immuno assay (11) was used for quantitation of M antigen in the extracts. Twenty ml 0.6% (w/vol) agarose (electroendosmosis (-m)) 0.16-0.19, Sea Kem Rockland, ME USA) in Tris/barbital buffer (0.07 M Tris and 0.02 M sodium barbital) containing 0.3 mM calcium lactate and 2 mM sodium azide, was mixed with various volumes of anti-type 12 serum and polyethyleneglycol 6000 (Kabo Sweden) (PEG) and poured in a 1 mm thick layer over a 205 × 110 mm glass plate. Circular wells taking 5 µl extract were cut 80 mm from the projected anode. A current of 3-4 V/cm was applied to the plate. The extract to be tested was then placed in the wells and the current was increased to 6 V/cm for 16 hours. The plates were washed in 0.9% NaCl and stained with Coomassie Blue.

Crossed Immuno-electrophoresis

Crossed immuno-electrophoresis (10) was performed using gel and buffer-systems as described above. The extract was first subjected to electrophoresis (20 V/cm for 40 min) in agarose without antiserum or PEG. Agar slips, 60 × 3 mm, were then cut out from the gel containing the separated extract, in the direction of the current and the slip was placed edge to edge with an agarose gel containing 1.5% anti-type 12 serum and 3% PEG. The extract components were forced into the antiserum-containing gel by application of a current of 6 V/cm for 16 hours (at an angle of 90° to the first

electrophoresis). Washings and staining were performed as described above.

Crude IgG Fc-Receptor Preparation

A crude preparation of IgG Fc receptor from type group A streptococci was obtained from a preparative electrophoresis as described (3). This preparation agglutinated Ripley-sensitized human red cells when diluted 1:20 000 or less (3).

Ion Exchange Chromatography

Ion exchange chromatography of hot hydrochloric acid extracts of type 12 group A streptococci was performed on CM-cellulose (Pharmacia Fine Chemicals Uppsala, Sweden) as described by Fox (5).

RESULTS

Identification of M12-Antigen Precipitate in Electro-immuno Assay

Anti-type 12 serum was incorporated in 1% agarose gel in concentrations from 1 to 3% (v/vol) and the PEG concentration was varied between 0 and 2% (w/vol). It was found that 1.5% serum and 2% PEG enabled a maximally distinct 'rocket' precipitate, when undiluted type 12 extract standard preparation was tested in the electro-immuno assay. The results were similar without the addition of PEG but the precipitates obtained were considerably less sharp. Electrophoresis at a voltage of 20 V/cm for 4 hours gave less distinct precipitates than those obtained at a voltage of 6 V/cm for 16 hours. At 6 V/cm, the precipitate formed extended about 1 mm from the application well (for accuracy, see below). Varying the dilution of the standard extract up to 1:4 resulted in a linear correlation between the concentration of the extract and the height of the major precipitate (Fig. 1). When using the standard extract, an additional precipitate was formed extending about 3 mm anodally from the application well.

In the following a concentration of 1.5% anti-type 12 serum and 2% PEG was used. Undiluted standard extract as well as 1:2 and 1:4 dilutions were included in each electro-immuno assay.

The major precipitate disappeared upon trypsinization of the standard extract (for procedure, see ref. 3) while the minor precipitate was unaltered. Testing of various fractions obtained from ion exchange chromatography of the standard extract showed that the components responsible for the major precipitate were eluted from the CM-cellulose column at pH 6.5. Formation of the major precipitate was completely inhibited on addition of anti-type 12 typing serum (Ca) in equal volumes to the standard extract but not by addition of anti-4, 28, 48 or 60 M-typing sera. Thus, the major

YERSINIA ENTEROCOLITICA AND Y ENTEROCOLITICA-LIKE BACTERIA ISOLATED FROM HEALTHY HUMANS IN NORWAY

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Kapperud, G *Yersinia enterocolitica* and *Y enterocolitica* like bacteria from healthy humans in
Norway Appl Microbiol scand Sect B 88 303-306 1980

Faeces samples from a total of 397 presumably healthy students in Norway were collected during April to September 1979. Twenty strains of *Y enterocolitica* and *Y enterocolitica* like bacteria were isolated from 10 (3%) of these students. Six students harboured 2-4 antigenically distinct strains. Of the total of 20 strains 14 were typical *Y enterocolitica* isolates representing five different serogroups (4 S 4 S 7 8 13-7 and non agglutinable). The remaining six strains were tentatively designated »*Y enterocolitica* like bacteria». They showed atypical reactions with respect to one or more of the following characters: fermentation of rhamnose, sucrose, cellobiose and sorbose. The strains recovered from healthy humans in this work were similar to strains previously isolated from wild living mammals, fish and water in Scandinavia. Nine strains produced enterotoxin at 22 °C but not at 37 °C.

Key words: *Yersinia enterocolitica*, humans, healthy carriers.

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Yersinia enterocolitica and *Y enterocolitica* like bacteria are *ex vivo* present in nature (15). This vast reservoir may be a considerable source of contamination for humans through drinking water, food and sexual contacts. Most of the strains prevailing in this reservoir are antigenically and biochemically *ex vivo* from those associated with the typical clinical manifestations of *Y enterocolitica* infection. However, there has been an increased awareness of the possible role of such »environmental» strains in atypical clinical syndromes (15). Numerous investigations have documented the presence of *Y enterocolitica* serogroup 3 and other serogroups with recognized clinical importance in different categories of human patients. However, little work has been done to evaluate the normal flora of *Y enterocolitica* and *Y enterocolitica* like bacteria (1, 2, 7). The present

study was undertaken to survey the presence of these bacteria in stool specimens from presumably healthy students in Norway.

MATERIALS AND METHODS

Collection of Faeces Samples

During the period April to September 1979 a total of 397 faeces samples were collected from six different groups (A-F) of presumably healthy students (age group 18-30 years) in Norway (Table 1). Faeces samples were collected on swabs and stored on a modified Stuart transport medium (16).

A. In the middle of April 18 samples were collected from high school students attending a winter camp in Haslingdal, central Norway. The drinking water was untreated in this area.

B. During the last week of April faeces samples were collected from 108 students of biology, University of

tates in the electro immuno assay with unadsorbed anti type 12 serum included to the gel. This finding made it possible to develop a method for the quantitation of M12 antigen using electro immuno assay.

Recent investigations indicate that M antigens are composed of repeating amino acid sequences (1). M antigens purified from hot hydrochloric acid extracts of all types investigated including type 12 group A streptococci show a marked heterogeneity in polyacrylamide electrophoresis (5, 6, 7). This property attributable to differing molecular weights of the M antigen fragments obtained by hot acid extraction has been called the »multiple banding phenomenon« each band so obtained contains compounds which are antigenetically identical making possible quantitation by immunochemical methods (5). The occurrence of multiple bands in polyacrylamide electrophoresis of M antigens does not imply that the bands are distributed »by chance« i.e. varying from one occasion to another. M proteins from different batches of cells of the same strain and also from unrelated strains of the same serotype have nearly identical patterns of mobility and each serotype has its characteristic distribution of bands (5). In the present paper crossed immuno-electrophoresis — which separates the antigens according to electrophoretic mobility — of three extracts of different type 12 group A streptococci gave a symmetrical precipitation peak in all experiments indicating a homogeneous electrophoretic mobility of the type 12 antigen fragments in *Lancefield* extracts. This conclusion was also supported by the fact that the bulk of M antigen from a hot hydrochloric acid extract was eluted from CM-cellulose in one major peak in agreement with the findings of Fox & Wittner (7). The homogeneous electrophoretic mobility of M12 antigens in *Lancefield* extracts indicated that the outcome of an electro immuno assay in which the extract of one strain is compared with the extract of another strain would not be erroneously influenced by differences in electrophoretic behaviour.

Like all other applications of the electro immuno assay technique the procedure described in the present paper is a simple, quick and reproducible way of determining individual components in a complex mixture. A greater sensitivity than with the capillary precipitin methods is probably achieved. Furthermore the qualitative estimation of precipitates makes it possible to avoid confusion with the other components present in a crude bacterial extract. It should be pointed out, however, that the amount of extractable M antigen does not necessarily reflect the amount present in

bacteria. Thus the extent to which the electro immuno assay can be used for comparison of the antigen quantities in different type 12 strains depends on standardization of the cultivation and extraction procedures. These problems are currently being analyzed.

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MATERIALS AND METHODS

Collection of Faeces Samples

During the period April to September 1979 a total of 397 faeces samples were collected from six different groups (A-F) of presumably healthy students (age group 18-30 years) in Norway (Table 1). Faeces samples were collected on swabs and stored on a modified Stuart transport medium (16).

A. In the middle of April 18 samples were collected from high school students attending a winter camp in Hallingdal, central Norway. The drinking water was untreated in this area.

B. During the last week of April faeces samples were collected from 108 students of biology, University of

Oslo one week after their Easter vacation. Most of the students spent these holidays in non urban areas with untreated drinking water.

C During the first two weeks of May, faeces were sampled from 72 medical students, University of Oslo. The samples from group B and C were obtained during the spring thaw in southern Norway.

D In June, 40 samples were obtained from zoologists attending field courses.

E The August samples were collected from 77 medical students, University of Bergen, the week subsequent to their summer vacation. During the summer, the students lived at widespread localities all over southern Norway. Most of the students had been exposed to untreated drinking water through mountain hiking and other outdoor activities.

F In September, 82 samples were collected from students at the Veterinary College of Norway, Oslo.

Processing Collected Material

Cultivation from the swabs was performed within one week on lactose/bromothymol-blue (Drigalski) agar and lactose/sucrose/urea (LSU) agar. The agar plates were incubated at room temperature (approximately 22 °C) for 48 hours. Sub cultures were made on lactose agar of all colonies showing morphological similarity to *Y. enterocolitica*. Urease positive strains were selected and a primary biochemical characterization was performed by the «three-tube method» described by Lassen (14). If the results indicated *Yersinia* the strains were subjected to further biochemical and antigenic characterization as specified previously (8).

Strains isolated from the same faeces sample were selected for study if they were antigenically and/or biochemically different.

RESULTS

Altogether, 20 strains of *Yersinia enterocolitica* and *Y. enterocolitica*-like bacteria were isolated from 10 (3%) of the 397 faeces samples from presumably healthy students in Norway (Table 1). The antigenic and biochemical characteristics of the isolates are presented in Table 2.

Characterization of the Isolates

Among the 20 strains isolated, 14 were typical *Y. enterocolitica* isolates (Table 2). Antigenically these strains were related to five different serogroups. One strain was non-agglutinable. Eleven of the *Y. enterocolitica* isolates could be ascribed to Wauters biotype 1 (18). The remaining three strains differed

They showed atypical reactions with respect to one or more of the following characters: fermentation of rhamnose, sucrose, cellobiose and sorbose. In the absence of a generally accepted taxonomy, these

TABLE 1 Isolation of *Y. enterocolitica* and *enterocolitica*-like Bacteria from Six Different Groups of Healthy Students in Norway

Group	Date	No. examined	Individuals harbouring <i>Yersinia</i> strains	
			No.	%
A	April	18	—	—
B	April	108	6	5.6
C	May	72	2	2.8
D	June	40	2	5.0
E	August	77	—	—
F	September	82	—	—
Total		397	10	3

TABLE 2 Antigenic and Biochemical Characteristics of *Y. enterocolitica* and *Y. enterocolitica*-like Bacteria from Healthy Students in Norway

Serogroup ^a	Biotype ^b	No. of strains
<i>Y. enterocolitica</i>		
4	1	1 ^c
5	1	2 ^d
6	1	2 ^d
6	1 \-	2
6	1 VP-	1
7-8	1	1 ^c
13-7	1	2
13-7	1 VP-	2 ^c
NAG	1 \-	1 ^c
<i>Y. enterocolitica</i> -like bacteria		
16	So- VP-	1 ^c
NAG	R +	1
NAG	S- VP-	2
NAG	C- So- VP-	1
NAG	R + S- C- VP-	1
Total		20

^a NAG = non agglutinable

^b According to Wauters' biotype scheme (18)

^c = Enterotoxin production at 22 °C (10)

^d = Both strains produce enterotoxin at 22 °C

Key to the symbols

\- = xylose non fermenting

VP- = Voges-Proskauer negative (at 22 °C and 37 °C)

So- = sorbose non fermenting

R + = rhamnose fermenting

S- = sucrose non fermenting

C- = cellobiose non fermenting

ns were tentatively designated »*Y enterocoli* like bacteria«

Range of Several Distinct Strains

Four of the ten students carrying *Yersinia* strains cultured two strains with distinct antigenic series. One person harboured three distinguishable strains. In a sixth case four antigenically distinct strains were isolated from the same faeces sample, namely serogroup 6 4 7-8 and non agglutinable.

■ person harbouring *Y enterocolitica* serogroup 6 in April was asked ■ take a second sample 6 weeks later. Two antigenically distinct strains (serogroup 16 and non agglutinable) were isolated from this second sample. The original serogroup 6 was not detected. No isolations were achieved from the second sample taken in June.

Clinical Significance

Four of the ten persons harbouring *Yersinia* strains claimed that they suffered from recurrent rheumatism and abdominal pain. These symptoms never could well be explained by psychical stress (12).

DISCUSSION

Examined students harboured *Yersinia enterocolitica* and *Y enterocolitica* like bacteria which were antigenically and biochemically similar to those previously isolated from wild living mammals in fish and water in Scandinavia (8 9 12 13). The students harboured several distinct strains. Deposited results were obtained by Alonso *et al* (11) who reported that humans inhabiting a rural area in Norway were healthy carriers of strains similar to those isolated from wild living small mammals in the same area.

The duration of the carriage was not determined. It is possible that the isolations may reflect a recent passage of the bacteria through the rural tract rather than a more permanent carrier.

Most of the students had been exposed to untreated drinking water. This may be an important source of contamination (13). Altogether 18 of the isolations now reported were made during the spring thaw when bacterial contamination of the drinking water is increased. However the results do not justify any statistically significant conclusion concerning a possible seasonal variation in the rate of *Yersinia* strains. Seasonal fluctuations in the frequency of human yersiniosis with a peak in the cold months of the year have been recorded from some European countries (3). A

similar trend was observed in an earlier study concerning the incidence of *Yersinia* strains in fish and water in Norway (12).

No clinical effect of the isolated strains could be established with certainty. Obviously examination of a very large material would be required to determine a possible clinical or sub-clinical role of the strains in question. When *Y enterocolitica* or *Y enterocolitica* like bacteria are isolated from diseased individuals the possibility of a causal connection should be examined critically. The isolation might well reflect an incidental contamination from the extensive environmental reservoir of these bacteria having no causal connection with the disease.

Nine of the twenty strains isolated in this work produced enterotoxin at 22 °C but not at 37 °C (10). Conclusive results have not yet been obtained concerning the clinical significance of the *Y enterocolitica* enterotoxin. Furthermore none of the isolates were invasive in a HeLa cell model (11).

Serogroup 3/biotype 4 and serogroup 9/biotype 2 responsible for the typical clinical manifestations of *Y enterocolitica* infections in Europe (15) were not recovered in this work. Nevertheless strains similar to those isolated have occasionally been reported in connection with atypical clinical syndromes (4 5 6 7 15 17). The presence of healthy carriers of such strains may be a potential hazard for susceptible hosts, for example immunologically deficient individuals through direct or indirect human transmission.

I want to thank the 397 students who contributed to the fulfilment of this study.

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IN VITRO ANTIBIOTIC SENSITIVITY TESTING OF *VIBRIO ALGINOLYTICUS*

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Larsen J L & Farid A F *In vitro* antibiotic sensitivity testing of *Vibrio alginolyticus* Acta path
microbiol scand Sect B 88 307-310 1980

V. alginolyticus from environmental sources of different geographical areas and some human pathogenic strains were investigated for their sensitivity to 29 different antimicrobial agents using the agar diffusion method. All strains were sensitive to Gentamicin Neomycin Sulfasodimidin Sulfamethoxazole Trimethoprim Rifamycin Nalidixan and Linco Spectin and all except one to Trimethoprim Tetracycline Chloramphenicol Nitrofurantoin and Tobramycin. Between 80 and 94% of tested strains were sensitive to Clindamycin Cephalotin Kanamycin Tylosin and Spiramycin while Ampicillin, Vancomycin and Novobiocin were effective to approximately 50%. A high percentage of resistance was found in Metocillin Carbenicillin and Lincomycin. All strains were resistant to Penicillin and Fucidin. No variation in sensitivity pattern between environmental strains isolated from different areas was detected. Great correlation was found among environmental and human pathogenic strains which could be helpful as guidance in therapy.

Key words: *Vibrio alginolyticus* antibiotic sensitivity testing *in vitro*

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The halophilic bacterium *Vibrio alginolyticus* was in marine coastal waters with highest valence during the bathing season when water temperature is high (4 6 8 10 12 16). It has lately been recognized as a human pathogen and lead from infection of the ear eye nose head wound and bronchial washings finger hand and toe leg foot and other unspecified sites of wounds exposed to marine environments (2 4 8 12 13). The infections are occasionally complicated by septicæmia.

Despite the increasing importance of *V. alginolyticus* from a medical point of view strains isolated from environmental sources are very seldom tested for their anti microbial susceptibility (5). Strains isolated from human septic infections are always investigated for their susceptibility to a number of known antibiotic agents. Generally *V. alginolyticus*

are certainly not extensively tested for anti microbial susceptibility in the world literature and no such investigation has been carried out in Denmark.

This paper presents the results of determinations performed on well-characterized *V. alginolyticus* strains isolated from different environmental sources in different geographical areas on the Danish coast. Some human pathogenic strains collected from different sources were tested under the same circumstances and the results were compared.

MATERIALS AND METHODS

Strains of *V. alginolyticus* from environmental sources comprised thirty marine strains fifteen estuarine and four sediment isolates (Larsen *et al.* (7) as well as 2 other sediment *Vibrio* strains. These strains were previously isolated from different bathing areas on the Danish

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TABLE 1 Number and Percentage of Sensitive (Highly Sensitive +++ and Moderately Sensitive ++) VAE and VAH strains

Antimicrob al/ug ^a	VAE 51 strains		VAH 7 strains		Antimicrobial/ug ^b	VAE 51 strains		VAH 7 strains	
	No of sens	%	No of sens	%		No of sens	%	No of sens	%
25 ²⁴	10	19	0	0	Chloramphenicol	30	58	7	100
Highly cl	100	19	2	28	Rifamycin ^c	30	51	7	100
10	24	46	3	42	Erythromycin	15	40	7	100
10	5	80	0	0	Lincomycin	15	11	1	14
100	13	25	1	14	Fucidin	50	0	0	0
30	47	92	7	100	Vancomycin	30	29	5	71
30	34	66	5	71	Nitrofurantoin	30	50	7	100
30	42	82	6	85	Nalidixan	30	51	7	100
30	51	100	7	100	Linco Spectin ^d	51	100	7	100
120	51	100	7	100	Tylosin ^e	150	38	6	85
30	35	68	5	71	Novobiocin ^e	100	24	6	85
250	51	100	7	100	Spiramycin ^e	200	40	7	100
methotazole									
25	51	100	7	100	Clindamycin ^f	25	48	5	71
52	50	98	7	100	Tobramycin ^g	40	50	7	100
30	50	98	7	100					

antibiotic concentration per disc

concentration in I U

new discs

compound of Spectomycin 200 µg + Lincomycin 19 µg

Nalidixan and Linco-Spectin whereas they are resistant to Penicillin and Fucidin

Actually strains with intermediate susceptibility comprised those originally evaluated as moderately sensitive (++) and slightly sensitive (+) When both the highly sensitive (+++) and moderately sensitive (++) strains are considered sensitive their total number and percentage are as given in Table 1 The results of the Table added Gentamicin and Rifamycin to the effective group of antibiotics shown in the figure as all VAE and VAH were sensitive Moreover 98% of VAE and all VAH were sensitive to Trimethoprim Tetracycline Chloramphenicol Nitrofurantoin and Tobramycin Ninety four per cent and ninety two per cent of VAE and seventy-one per cent and all VAH were sensitive to Clindamycin and Cephalotin respectively

Antimicrob

the strain

ed S

Novobiocin were effective against approximately 24 per cent of strains tested Actually all strains are highly sensitive to Sulfasodimidine This is a thionamide not presented in Fig 1

DISCUSSION

A correct strategic and tactic use of chemotherapy against bacterial infections requires reliable information on the efficiency of drugs against the causal bacterial species Such data are gained from bacteriological laboratories performing antibiotic sensitivity testing (11) The preliminary or basic knowledge about the action of different antimicrobial agents on the species of bacteria encountered is greatly helpful No such data are available in case of the halophilic bacterium *Vibrio alginolyticus* as it is a relatively new species considered pathogenic to man

Twenty nine different antimicrobial agents were used to investigate the susceptibility of *V. alginolyticus* strains The *in vitro* testing was performed by the agar diffusion method which in a recent study (5) proved to be applicable for this group of organisms

The present study demonstrates a high incidence of sensitivity to different agents All strains of VAE and VAH were sensitive to Gentamicin Neomycin Sulfasodimidine Sulfamethoxazole Trimethoprim Rifamycin Nalidixan and Linco-Spectin All strains

coasts and were characterized by their biochemical behaviour (7) The human pathogenic strains were obtained from Dr D G Hollis CDC, Atlanta, Georgia, USA (strains 2300/79, 1424/79, and 1586/79), Dr Mary L. Fried, Tucson Medical Center, Tucson, Arizona, USA (T M C strain) and Dr A. von Graevenitz, Yale University School of Medicine New Haven Connecticut, USA (Yale strain) The last two strains were previously described (15, 17 respectively) Two human pathogenic strains previously isolated in Denmark (9) were also examined Unless otherwise indicated, *V. alginolyticus* strains from environmental sources are called VAE, whereas human pathogenic strains are called VAH

The antibiotic discs or tablets of 29 anti-microbial agents used in this study are commercially available (AB-Biodisk, Solna, Sweden, and A/S Rosco, Neo-Sentab[®], Taastrup, Denmark) and are listed in Table 1

Anti-microbial sensitivity was determined by the standard agar diffusion method (3) though the organisms were grown in trypticase soy broth (BBL) with 1% sodium chloride added and the cultures were applied to

Mueller-Hinton agar (Difco) plates with addition of sodium chloride (0.5%), for susceptibility testing

Interpretation of results was performed by measuring the diameters of the inhibition zones in millimeters, and the readings were compared with corresponding manufacturing tables Mostly four grades were evaluated: highly sensitive (+++), moderately sensitive (++) slightly sensitive (+), and resistant

RESULTS

The results are presented in Fig. 1, illustrating the zone diameters and accepted breakpoints between resistance and susceptibility for all antibiotics used Strains giving inhibition zones that could not be evaluated as highly sensitive were considered intermediately susceptible and presented in the area between the solid and broken lines The figure shows that all VAE and VAH strains are sensitive to Neomycin, Sulphamethoxazole-Trimethoprim

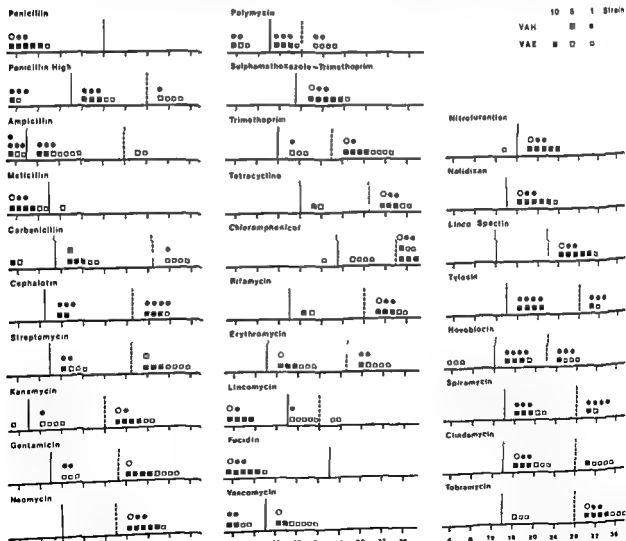


Fig. 1 Inhibition zones for VAE and VAH strains with the employed antibiotics. Solid lines separate sensitive from resistant strains. Broken lines divide highly from intermediately sensitive strains.

AN *IN VITRO* EVALUATION OF TWO COMMERCIAL BLOOD CULTURE MEDIA - SUPPLEMENTED PEPTONE BROTH AND SUPPLEMENTED TRYPTONE SOYA BROTH

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Sandven P & Høiby E A An *in vitro* evaluation of two commercial blood culture media - supplemented peptone broth and supplemented tryptone soya broth Acta path microbiol scand Sect B 88 311-315 1980

Two commercial blood culture media - supplemented peptone broth and supplemented tryptone soya broth - were compared by an *in vitro* method. The two media were inoculated with microorganisms suspended in fresh human blood in an attempt to mimic the clinical situation. The two media differed completely in their ability to support the growth of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. Macroscopic evaluation of the blood culture medium during the incubation period was difficult when using supplemented tryptone soya broth in contrast to supplemented peptone broth. The former medium remained turbid whether microorganisms were growing or not. No other important differences between the two media were detected. Our findings indicate a need for both *in vitro* and *in vivo* studies to evaluate blood culture media and also a need for quality control of microbiological diagnostic devices before they are released for clinical use.

Key words: Blood culture media, *Neisseria meningitidis*.

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The detection of bacteraemia is one of the most important tasks in clinical bacteriology. A number of clinical and *in vitro* studies have shown that the type of blood culture medium used influences the recovery of microorganisms. Ideally all such media which are commercially available ought to be evaluated in clinical studies before being released for general sale, but because such trials are time-consuming and expensive this is not the case today. An alternative is to evaluate blood culture media by *in vitro* experiments. It is however necessary to mimic the clinical situation as closely as possible. We have tried to do this by using fresh human whole blood for the experiments. The two blood culture devices compared in this study are suitable for the cultivation of only two ml of blood and do not according to accepted recommendations (1) only be used for pediatric blood cultures. In spite of these recommendations these blood culture devices are still commonly used in Norway today.

MATERIALS AND METHODS

Blood culture media: Vacutainer blood culture tubes with

modified experimental medium containing 1.2% gelatin and lacking agar (STSBG) were obtained from Terumo Corporation, Japan. The ingredients of SPB and STSB are shown in Table 1. All tubes contained 18 ml of medium determined for an inoculum of approximately 2 ml of blood. The concentration of sodium polyanethol sulfonate (SPS) in SPB was 0.025% and in STSB 0.035%.

Microorganisms: Recent clinical isolates obtained from the diagnostic microbiology laboratory of the institute were used. Strains isolated from blood cultures were omitted in order to avoid selection of strains already having grown in one of the blood culture media we were testing. The clinical isolates used were 2 strains each of *Escherichia coli*, *Proteus mirabilis*, *Klebsiella* sp., *Pseudomonas aeruginosa*, *Haemophilus influenzae*.

except one are sensitive to Trimethoprim Tetracycline Chloramphenicol Nitrofurantoin and Tobramycin These results are in agreement with investigations sporadically performed on isolated human pathogenic strains reviewed (1 15) However sulfa drugs were not tested in most reports

Cephalotin was 100% effective against VAH and 92% against VAE This was also found in two human pathogenic strains recently isolated in USA (14) while resistant strains were previously reported (10 12 13 15) Two tested strains of VAH were resistant to Clindamycin which is also recorded by Schmidt *et al* (14) but not encountered in other papers

About 70% of the strains were sensitive to Polymyxin (Colistin) It was previously reported to be effective with the exception of the recent two strains isolated (14) Although reports deal with high numbers of Ampicillin resistant strains (5 10 12 13 15 17) a moderate incidence of Ampicillin Vancomycin and Novobiocin resistant strains could be detected in the VAE and VAH strains

A high percentage of resistance was found to Meticillin Carbenicillin and Lincomycin while all strains show resistance to Penicillin and Fucidin Especially results of Carbenicillin and Ampicillin resistance are well documented by most authors (10 12 13 14 15 17)

In conclusion these findings show great correlation in sensitivity patterns among VAE and VAH strains and no variation in environmental strains isolated from different geographical areas The results could offer a basic information about the broad action of various antimicrobial agents on *V. alginolyticus* *in vitro* and might serve as a guidance in therapy

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TABLE 3 Growth Pattern of Identical Inocula of *Pseudomonas aeruginosa*, *Bacteroides fragilis*, *N. meningitidis* and *N. gonorrhoeae* in the Blood Culture Media Tested

Organism	No. of organisms in the inoculum	Blood culture medium	Growth ^a by subculture in parallel blood culture tubes after	
			18 hours	42 hours
<i>Pseudomonas</i>	4	SPB STSB	+++ ---	+++ +++
<i>Pseudomonas</i>	4	SPB STSB	+++ +++	+++ +++
<i>Fragilis</i>	6	SPB STSB	---+ +++	+++ +++
<i>Fragilis</i>	12	SPB STSB	+++ +++	+++ +++
<i>Gonorrhoeae</i>	12	SPB STSB	+++ ---	+++ ---
<i>Gonorrhoeae</i>	2	SPB STSB STSBG	--- --- ---	---+ --- ---
<i>Meningitidis</i>	14	SPB STSB STSBG	+++ --- ---	+++ --- ---
<i>Meningitidis</i>	6	SPB STSB STSBG	+++ --- +++	+++ --- +++
<i>Meningitidis</i>	210	SPB STSB STSBG	++ -- --	++ -- --
<i>Meningitidis</i>	36	SPB STSB STSBG	---+ -- --	+ -- --
<i>Meningitidis</i>	180	SPB STSB STSBG	---+ ++ ++	+ ++ ++
<i>Meningitidis</i>	18	SPB STSB STSBG	++ -- +	++ ++ +
<i>Meningitidis</i>	32	SPB STSB STSBG	---+ -- ---	---+ -- ---
<i>Meningitidis</i>	2	SPB STSB STSBG	---+ --- ---	---+ --- ---
<i>Meningitidis</i>	16	SPB	+++	+++

TABLE 1 *Formulae of SPB and STSB Blood Culture Media*

Ingredient	SPB (grams per liter)	STSB
Yeast extract	9.4	5.0
Tryptic digest of casein	4.7	
Enzymatic hydrolysate of meat	4.7	
Tryptone		17.0
Soya peptone		3.0
Meat extract		3.0
Liver digest		1.0
Gelatin	12.0	
Sodium bicarbonate	2.2	
Dextrose	2.5	
Glucose		2.5
Sodium chloride	4.0	5.0
K_2HPO_4		2.5
Adenine sulfate	0.01	
Magnesium sulfate	0.2	
Sodium polyanethol sulfonate	0.25	0.35
Cystein HCl	0.26	0.45
Sodium phosphate (dibasic)	0.14	
Para aminobenzoic acid	0.05	0.05
Proline	0.05	
Glutamic acid	0.05	
Nicotinamide adenine dinucleotide	0.0025	
Coccarboxylase	0.0003	
Vitamin B 12	0.0001	
Menadione sodium bisulfite	0.0002	
Hemin	0.005	0.005
Guanine HCl	0.0003	
Purified agar		0.01

Staphylococcus aureus, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus faecalis*, *Streptococcus viridans*, *Bacteroides fragilis* ssp. *fragilis*, *Candida albicans*, *Neisseria gonorrhoeae*, and 6 strains of *Neisseria meningitidis*.

Preparation of the inoculum: Human blood was obtained by venipuncture immediately prior to use from two healthy donors (A.H. and P.S.) who had not received antibiotics during the previous months. For each

blood sample, 2.5 ml was centrifuged at 1000 g for 10 min. The supernatant plasma was removed and the cells were washed in 0.9% phosphate buffered saline (PBS) pH 7.4. The optical density of each suspension was measured and adjusted to give an optical density of 0.6 at 620 nm. The suspension was afterwards serially diluted to give a suitable number of microorganisms per ml of PBS. The size of the inoculum was chosen to mimic a low grade bacteraemia and at the same time to avoid such small numbers of microorganisms that an uneven

distribution in the tubes resulted. The exact number of microorganisms used for inoculation was calculated by determining the average colony forming units from plating 0.1 ml samples on three chocolate agar plates. Colonies were counted after 48 hours of incubation at 37 °C.

One ml of the suspension of microorganisms was inoculated into each of the portions of fresh blood. The tubes containing the suspension of blood and microorganisms were then thoroughly mixed. One test organism was dealt with at a time in order to inoculate the blood culture tubes as fast as possible. The time used for each organism never exceeded five minutes.

Inoculation and evaluation of the blood culture media: The rubber stopper of each blood culture tube was cleansed with 70% ethanol. Two ml of the mixture of blood and microorganisms was inoculated into the blood culture tubes. Three parallel tubes of each brand (SPB and STSB) were inoculated in a random order. Two parallels were prepared for two of the meningococcal strains. Parallel tubes of STSBG were inoculated for one *N. gonorrhoeae* strain and four *N. meningitidis* strains. The blood culture tubes were turned upside down to mix the contents and then vented by the use of venting unit of respective brand. The media were incubated in air at 37 °C. At 18 h, 42 h, 66 h, 90 h and after 7 and 14 days of incubation the tubes were inspected for evidence of macroscopic growth and subcultured after thorough mixing. Six drops of the medium were removed through the venting unit, plated on chocolate agar and incubated at 37 °C with 5% CO₂ for 48 hours except for *B. fragilis* which was incubated in a Gas Pak® jar.

Agar diffusion test: The sensitivity of 3 *N. meningitidis* strains to SPB, STSB, STSBG and SPS was tested using an agar diffusion test. PDNI - Antibiotic Sensitivity Medium AB Biodisk Stockholm (PDNI medium) was used for the tests. The *N. meningitidis* strains were grown on chocolate agar for 18 hours and a suspension containing approximately 10⁴ colony forming units per ml was made in 0.9% NaCl. The surface of the medium was seeded with 5 ml of this suspension giving a dense

TABLE 2 *No. of Viable Microorganisms Used to Inoculate Each Blood Culture Tube*

	Inoculum ¹
<i>E. coli</i>	4/2
<i>Klebsiella</i> sp.	4/4
<i>P. mirabilis</i>	8/8
<i>H. influenzae</i>	4/2
<i>Staph. aureus</i>	110/6
<i>S. pyogenes</i>	6/4
<i>S. faecalis</i>	12/22
<i>S. viridans</i>	12/16
<i>S. pneumoniae</i>	16/14
<i>C. albicans</i>	56/60

¹ Colony forming units inoculated per blood culture tube. Two strains of each species were examined.

rich blood culture media contain SPS Eng & and (4) have shown that SPS may be harmful to *meningitidis* and *N gonorrhoeae* strains. This was eliminated by adding 1.2% gelatin to the blood culture media (3). Gelatin is incorporated in STSB but not in STSB. It was therefore thought that the lack of gelatin in STSB was the reason for the difference between SPB and STSB as to the growth of *Neisseria* sp. The experimental medium (STSBG) from Terumo (STSB containing 1.2% gelatin and lacking agar) did not

show growth in SPB (Table 3). Other factors in addition to toxicity of SPS to *Neisseria* sp. must therefore be taken into consideration to explain the poor growth of *meningitidis* and *N gonorrhoeae* in STSB. The diffusion test in agar indicates that SPB contains substances probably lacking in STSB that support the growth of *N meningitidis*. In addition, STSB seems to contain substances that inhibit the growth of *N meningitidis* since a zone of reduced growth was produced around the well containing STSB. A part of this effect may be due to SPS. The zone of inhibited growth was, however, also noted when testing STSBG. In this medium the toxicity of SPS is probably neutralized by the gelatin content of the medium. It is therefore likely that other constituents in STSB may also contribute to the toxic effect seen.

The two media also differ as to the content of dissolved gases and pH (Table 4). The PO_2 is slightly lower in SPB than in STSB (20 mm Hg and 12 mm Hg, respectively). The PCO_2 is also higher in SPB than in STSB (57 mm Hg and 6 mm Hg, respectively).

Both SPB and STSB are rather complex media containing a number of undefined biological substances such as soya peptone, tryptone etc. Evalua-

tion of each component and combinations of them as to their effect on *N meningitidis* may therefore be complicated.

The method of preparing bacterial suspensions in blood exposes the meningococci to SPS without gelatin (2). This factor operates equally on all parallels and cannot explain the differences seen.

Although bacteraemias caused by *N meningitidis* are not rare in Norway, it is doubtful if the important difference between the two blood culture media tested would have been detected in a clinical study. This might indicate a need for both *in vitro* and *in vivo* studies to evaluate blood culture media.

This study does reveal a need for thorough quality control of microbiological diagnostic devices before they are released for clinical use.

We thank Harald Goldstein, Institute of Economics, University of Oslo, for conducting the statistical analysis of the data presented in this study.

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but not completely confluent growth. The plates were allowed to dry for 30 minutes. Wells (7 mm in diameter) were made in the medium and 0.1 ml of the substance to be tested was placed in the well. The results were read after 18 hours of incubation in 5% CO₂ at 37 °C.

Analysis of soluble gases and pH. Samples were taken by syringe and needle from two parallel blood culture tubes each of SPB and STSB and tested immediately for PCO₂, PO₂ and pH on a Corning pH Blood Gas Analyzer 165 before and immediately after inoculation with 2 ml fresh blood.

RESULTS

The inocula used for the *E. coli*, *Klebsiella P. mirabilis*, *H. influenzae*, *Staph. aureus*, *S. pyogenes*, *S. faecalis*, *S. viridans*, *S. pneumoniae* and *C. albicans* strains are shown in Table 2. For these microorganisms no difference was observed between the two media tested. The *H. influenzae* and *S. viridans* strains and one *C. albicans* strain were detected on subculturing after 42 hours of incubation. The other strains were detected after 18 hours of incubation. The results obtained on subculturing for the other microorganisms used are shown in Table 3. For the whole study no blood culture negative at 42 hours showed growth on subculture later on.

Small differences between the two blood culture media are noted for one of the *Pseudomonas* strains used and for one of the *Bacteroides* strains. For the *Neisseria* strains however the differences between the two media are statistically highly significant ($p < 10^{-6}$). SPB supported the growth of all strains tested but only one of the eight strains was able to grow in STSB. The experimental medium containing 1.2% gelatin and lacking agar STSBG were compared with the other two media (SPB and STSB) using 4 *N. meningitidis* strains and 1 *N. gonorrhoeae* strain (Table 3). Only for two strains was growth detected in STSBG.

The agar diffusion test on PDM medium using 0.1 ml of 0.025% SPS or 0.035% SPS in the well yielded a zone of no growth around the well. The diameter of the zone varied from strain to strain but all meningococcal strains investigated were inhibited by SPS. Around the well containing SPB a zone of enhanced growth of *N. meningitidis* was found in contrast to growth of smaller colonies on the rest of the agar surface. A zone of markedly inhibited growth of *N. meningitidis* was found around the wells containing STSB or STSBG. The size of this zone was approximately equal to the zone of no growth found around the well containing 0.1 ml of 0.035% SPS. The pH, PCO₂ and PO₂ of SPB and STSB before and after the addition of fresh blood are shown in Table 4.

TABLE 4. *Analysis of Soluble Gases and pH in SPB, STSB*

Blood culture medium	Before addition of blood			Immediately after addition of blood		
	pH	PO ₂ *	PCO ₂ *	pH	PO ₂	PC
SPB	7.1	20	57	7.2	34	3
STSB	7.0	12	6	7.0	30	1

* mm of Hg

The tubes inoculated with blood and microorganisms were compared with a negative control tube containing only blood from the same donor. Evidence of macroscopic growth. For most of the microorganisms used macroscopic growth was easily detected in SPB. This medium remained clear when negative on subculture. Macroscopic signs of growth of *N. meningitidis* and *N. gonorrhoeae* were usually detected 2 or more days after growth had been detected by subculturing. When STSB was used macroscopic evaluation was very difficult. The medium remained turbid whether microorganisms were growing in the medium or not. Macroscopic growth was detected only when visible colonies were present in the medium if hemolysis occurred or if massive opacity resulted.

DISCUSSION

The present study does of course suffer from limitations inherent when using an *in vitro* method. The number of strains tested for each species are except for the meningococci too small to permit a valid conclusion as to the growth supporting ability of the two blood culture media and this part of the study was meant as a screening only. The strains used were freshly isolated strains from clinical material. It is however obvious that bacteria from patients with septicæmia may be more difficult to grow than strains already having been isolated on bacteriological media. Taking these limitations into consideration it is probable that only comparatively large differences between the two blood culture media tested can be detected by an *in vitro* method. In spite of this we have been able to detect important differences between them. Macroscopic evaluation of SPB was usually easy in contrast to STSB. Using the latter is therefore likely to impose a lot of extra work on the bacteriological laboratory since repeated subculturing and microscopical examination will be necessary to ensure that there is no growth in the media.

RESISTANCE TYPES IN *ESCHERICHIA COLI*

II Transfer of Ampicillin Resistance

PER SØGAARD

Statens Seruminstitut, Regional Laboratory Odense University Hospital Odense Denmark

Sogaard P Resistance types in *Escherichia coli* II Transfer of ampicillin resistance Acta path microbiol scand Sect B 88 317-322 1980

The ability to transfer resistance traits was investigated in 49 *E. coli* strains isolated from clinical specimens. The strains were divided into three groups according to sensitivity to penicillin derivatives: group 1 contained 8 ampicillin-carbenicillin sensitive (A^s/Ca^s) strains, group 2 contained 16 ampicillin-resistant-carbenicillin sensitive (A^r/Ca^s) strains and group 3 contained 25 ampicillin-uterbecillin resistant strains. In group 3, 17 strains could transfer A resistance (range of transfer frequency was 10^{0.0} to 10^{7.5}). The 16 strains in group 2 did not transfer A resistance more often than mutants arose from the recipient (10^{-8.8}). The mutants of the recipient selected on A plates were A^r/A^s/cephalothin resistant exactly as the strains in group 2. The A resistance in group 3 was probably based on plasmids and that of group 2 was based on mobilizable plasmids and/or chromosomal resistance. The frequencies of transfer of sulphonamide, tetracycline and streptomycin resistance of the strains in groups 1 and 2 that did transfer were in the same range as the frequencies of transfer in group 3.

Key words: *Escherichia coli*, resistance types, transfer of ampicillin resistance.

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Transferable and non transferable antibiotic resistance in enteric bacteria was investigated by Bergheim *et al.* (1972). They found that ampicillin resistance (A^r) could be transferred in about 25% of A resistant (A^r) strains. Highly resistant strains could often transfer A^r and mediated resistance to very high concentrations of the antibiotic in the recipient.

Four strains of *E. coli* can be divided into ampicillin sensitive (Ca^s) and Ca^r strains. Sogaard *et al.* (1979) found the former group less resistant to A and more resistant to cephalothin (Ce) than the latter one.

Therefore an investigation was carried out to determine whether the frequency of transfer of A^r is different in the two groups.

MATERIALS AND METHODS

A Strains

The 49 clinical isolates were divided into the 3 groups shown in Table 1. The strains appear in the same order in Tables 2, 3 and 4 in this paper as in Sogaard (1979).

B Media

Medium 1: Modified Conradi Drigalski plates containing nalidixic acid (Nal) 25 µg/ml.

The modified Conradi Drigalski plates have the following composition (Gaarslev 1980): demineralized H₂O 1 liter, peptone (A/S Orto) 27.70 g, yeast extract (A/S Orto) 10.00 g, sodium dodecylbenzolsulfonate (Marani) 1.00 g, thiosulphate 2 ml 50%, bromthymol blue 10 ml 1%, sodium dodecylbenzolsulfonate (Marani) 1.00 g.

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MATERIALS AND METHODS

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TABLE 1 *E. coli* Strains Used

Strain	Phenotype	Source
Clinical isolates group 1 (n=8)	A s/Ca s	Sogaard (1979)
Clinical isolates group 2 (n=16)	A r/Ca s	Sogaard
Clinical isolates group 3 (n=25)	A r/Ca r	Sogaard
K 12 W3132 <i>nal</i> ^a	F Met ⁻ Nal ^r	J Orskov
K 12 1100 F Km	F Km ^r	K Nordstrom

Abbreviations: A = ampicillin Ca = carbenicillin Nal = nalidixic acid Km = kanamycin s = sensitive r = resistant Met = methionine ^a = a nalidixic acid resistant mutant of strain W3132 (Sogaard 1973)

Brøste P A/S DK 1415 København K) 1 ml 5% pH adjusted to 7.7-7.8

As appropriate the plates were supplemented by sulphonamide (Su) 100 µg/ml tetracycline (T) 20 µg/ml streptomycin (S) 20 µg/ml chloramphenicol (C) 20 µg/ml or A 10 15 20 or 25 µg/ml. The plates with Su were peptone free.

Medium 2 The plasmid transconjugants were tested on methionine (Met) free and Met containing plates of the following composition: K_2HPO_4 7 g KH_2PO_4 3 g sodium citrate 3 H_2O 0.5 g $MgSO_4$ 7 H_2O 0.1 g $(NH_4)_2SO_4$ 1 g glucose 5 g agar 2.5 g H_2O 1000 ml. The Met plates contained 10 µg Met/ml.

Medium 3 Resistance plates (Casals & Pedersen 1980 p. 6-7)

Medium 4 5% blood plates (Laurup *et al.* 1979)

Medium 5 Infusion broth

Medium 6 Water agar (2.5% Bacto agar in distilled H_2O)

Medium 7 Agar plates (concentrated ox broth with agar 1.7%)

All substrates were obtained from the substrate department Statens Serum Institut Copenhagen.

All cultures were done at 35 °C.

C Direct Transfer Assay

The transfer was performed as described by Bergfors *et al.* (1972). The donors were grown in medium 5 to a density of approx. 10^8 CFU/0.1 ml (mean log CFU/0.1 ml 7.98 s.d. 0.48 range 6.0-8.7). The recipients were grown in medium 5 with shaking to a density of approx. 10^8 CFU/0.1 ml (mean log CFU/0.1 ml 7.88 s.d. 0.45 range 6.8-8.5). Donor culture (0.1 ml) was mixed with 1.0 ml of recipient culture. The mixture was incubated at 35 °C for 2 h before 10 ml of medium 5 was added. After overnight incubation the broth was diluted 10^2 , 10^4 and 10^6 0.1 ml of undiluted and 0.1 ml of the 3 dilutions were spread on appropriate plates of medium 1. Transfer of resistance to each antibiotic was tested on separate plates. If any colonies appeared on the plates two were used for resistance determination on medium 3 with the agar diffusion technique (Casals & Pedersen 1980) to Su T S C Ce A Ca and Nal to confirm the transfer of resistance traits. Material from the colonies was also tested on Met free and Met-containing medium 2 to ensure that the conjugants had the phenotype Met⁻

of the recipient. All the donors could grow on Met⁺ medium 2.

From an overnight culture of donor and recipient 1 ml was spread as control on the same plates as the mix culture.

The number of CFU in the mixed culture determined both on medium 1 and medium 4. 1 difference between the two counts was the number of donors in the mixed culture.

D Mobilization Assay

This assay was done in two steps.

Step 1 The *E. coli* strain K 12 1100 F Km was used as donor of the F factor. Nine clinical isolates of group (A r/Ca s) were recipients. The transfer was done described in C. A r/km^r conjugants were isolated on medium 1 with A 20 µg/ml and kanamycin 20 µg/ml but without Nal.

Step 2 The Km^r clinical isolates from step 1 were used in a direct transfer with *E. coli* K 12 W3132 *nal* recipient as described in C.

E Resistance Test

This test was made as an agar diffusion test (Casals & Pedersen 1980) using medium 3 and antibiotic disc (Neosensitabs®). A strain with diameter of inhibition zone less than 23 mm was called resistant to the antibiotic in question.

F Colicin Production Test

If the recipient (W3132) was eradicated from the mixed cultures of donor and recipient the donor was suspected of producing colicin. This was shown as follows. A colony of the donor was grown on an agar plate (medium 7). An overnight culture of the recipient (W3132) was made. 2 ml of hot liquid medium 6 was mixed with 2 ml of recipient culture and poured over the agar plate with the donor colony. The plate was incubated overnight at 35 °C. The formation of a clear zone without growth around the donor colony showed the colicin production.

G Materials

The following antibiotics were used in the plate: Aminobenzylpenicillin D and carbenicillin (Fugacilin®), Astra A/B Sodertälje Sweden. Sulfadimidin (Danmark)

121611 København V
 121611, DAK Dihydrostreptomycin sulfat, Nord sk
 121611 DK 2100 København Ø Kloramfen col DAK
 121611 and Sterling Winthrop A/S DK 2100 Kø
 121611 Ø Kanamycinsulfat, Lundbeck DK 2500
 121611

RESULTS

2 shows the frequency of transfer of A R. In
 3 (A r/Ca r) 17 of 23 strains were able to
 fer their A R usually with high frequency
 strain produced colicin (col) and one was

group 2 (A r/Ca s) 5 strains were omitted
 to col formation or Nal^r. Among the

E 2 Frequency of Transfer of Amp^r Cl^r Res^s
 among 41 Amp^r Cl^r Resistant E. coli Strains

Strain	A r/Ca s (group 2) n = 16	A r/Ca r (group 3) n = 25
C		17
		57
		75
i		34
		27
Nal ^r		col
		-15
		-07
		-62
79		-61
		-60
col		Nal ^r
col		00
		00
Nal ^r		39
Nal ^r		-03
		26
		-04
		-22

2 is expressed as log₁₀ the ratio between the
 number of A^r recipients and the number of donors
 in the presence of tetracycline S streptomycin
 chloramphenicol D no resistance to Su T S or C
 to transfer observed colicin produced by the
 as legend to Table 1

remaining 11 strains only one (resistance type SuS)
 could apparently transfer its A R in the first
 attempt. The transfer frequency of this strain was
 very small 10^{-7.9} (Su or S R was not transferred).
 The difference in frequency between the two groups
 is significant by Wilcoxon's test (p < 0.01). The
 experiment was repeated in the same manner for all
 11 strains except the two strains that transferred
 SuTS and TR respectively (Table 3). One (resis-
 tance type O) out of these 9 strains apparently
 transferred A R (frequency 10^{-7.4}). The strain
 mentioned above did not transfer A R in this
 experiment.

When 2 × 10⁹ bacteria of the recipient W3132
 were spread on medium I with A (20 µg/ml) + Nal
 (25 µg/ml) 3 colonies of A^r bacteria were
 observed (frequency 10^{-8.9}). These three mutants
 were Ca s and Ce r as the strains in group 2. The
 frequency of S^r mutants of the recipient was also
 10^{-8.8}. No Su^r, T^r or C^r mutants were observed.
 Nal^r mutants of the donors were observed with a
 frequency of 10^{-7.5}.

As mentioned by Søgaard (1979) 2 strains in
 group 3 had the same resistance profile as the
 bacteria in group 2, i.e. less resistant to Ca than to A
 and very resistant to Ce. No A R transfer was
 observed with these 2 strains. One could transfer
 Su R (frequency 4.3) and the other produced
 colicin (Table 3).

Table 3 shows the transfer of the resistance traits.
 Su^r, T^r, S^r and C^r Su R was transferred from 2 (6) 1
 (7) and 9 (16) strains in groups 1, 2 and 3
 respectively. Figures in parentheses show the
 number of strains tested. T^r was transferred in 1
 (2) 2 (3) and 5 (8) cases in groups 1, 2 and 3
 respectively. The corresponding numbers for S^r
 were 2 (6) 1 (7) and 1 (16). C^r was transferred
 from 5 (8) strains in group 3.

The transfer frequency of A R of each strain in
 group 3 was approx. the same as the transfer
 frequencies of Su^r, T^r, S^r and C^r (as applicable).
 corresponding to simultaneous transfer of all
 resistance traits (Table 2 and 3).

In group 2 none of the two strains that
 transferred SuTS and TR respectively transferred
 A R.

The mobilization assay was done with the 9
 group 2 strains mentioned in the second paragraph.
 The results are shown in Table 4. A R could be
 transferred from 4 strains. The highest frequency
 was 10^{-4.1}. The bacteria of the observed colonies on
 the A plates were A r/Ca s/Ce r and had no other
 resistance traits except one which showed T^r. The
 transfer frequency of T^r in this mating was very high
 (10^{-1.0}). Three out of the 5 strains with resistance
 types SuTS and SuS could transfer Su^r, T^r and/or

TABLE 3 *Transfer of Resistance Traits (Ampicillin Excluded) According to Resistance Type among 33 E. coli Strains*

Resistance type	A s/Ca s (group 1) n = 8	A r/Ca s (group 2) n = 8	A r/Ca r (group 3) n = 17
SuTSC			SuTSC (-0.3 -0.5 -0.6 -3.7) ~ SuTSC (-6.9 -6.3 -5.7 -6.3) SuTSC (-6.8 -7.3 -7.3 -7.4)
SuTS	Nal r SuS (-3.8 -4.0) Nal r T (-6.8)	SuTS (-3.1 -2.7 -4.9) ~ T (-1.3) Nal r	SuTS (-3.6 -3.4 -5.3) ~ T (-2.5) ~ col
SuSC			~ SuSC (-1.5 -1.6 -1.6) SuSC (-0.7 -0.6 -0.5) ~
SuS	~ SuS (-3.9 -4.0) ~ ~	~ ~ ~ ~	~ SuS (-5.8 -5.5) Su (-4.3) SuS (-5.0 -5.0)

Abbreviations See legend to Table 2

Parentheses show the transfer frequency of corresponding resistance trait

S R None of the colonies formed showed A R 12
out of 22 colonies tested showed Km R

TABLE 4 *Transfer of Resistance Traits in Mobilization Assay of 9 A r/Ca s E. coli Strains*

Resistance type	
SuTS	SuTSA (-6.6 -1.0 -6.3 -6.8) Nal r
SuS	SuS (-6.4 -6.4) SuSA (-5.6 -5.7 -4.1) A (-8.0) ~
O	col col A (-7.9) Nal r Nal r ~ ~ ~

~ strain not tested
See legend in Table 3

DISCUSSION

The results suggest that the A R seen in group 1 was based on plasmids while the A R in group 2 were not transferred in the direct matings (Table 2). The other resistance traits could be transferred from some of the strains in groups 1 and 2 (Table 3). The transfer frequencies of these resistance traits in groups 1 and 2 were as high as the frequencies in group 3.

From Fig. 3 of the paper by Bergfors *et al.* (1977) it can be concluded

- A r strains with high resistance can often transfer their resistance and confer a high degree of resistance in the recipient.
- A r strains with lower resistance cannot usually transfer their resistance.

The results in this paper confirm these findings and show that the group of A r/Ca s E. coli strains comprises or at least belongs to the strains mentioned above under b.

Group 3 (A r/Ca r) could transfer A R very often and had a high degree of resistance. This agrees well with production of class III β -lactamases as these enzymes are plasmid mediated and have a high activity against A (Richmond & Sykes 1973). Plasmids with genes for class III β -lactamases are known to be widespread.

In the matings with donors of group 2 A r

us were found with a small frequency in two strains. These colonies consisted of A-r/Ca-s/Ce-r. The overall frequency for A-R "transfer" was $10^{-9.0}$. This frequency does not differ from the frequency of spontaneous A-r ($10^{-8.9}$) obtained in the control experiments. These mutants were A-r/Ca-s/Ce-r, i.e. same phenotype as the strains in group 2. Therefore it is a possibility that the group 2 strains arose by mutation. The mobilization assay further showed that A-R transfer from some of the strains in group 2 could be established by means of a P factor (Table 4). The frequencies were small, though showed transfer of A-R with a frequency of $10^{-4.1}$. This differs from the frequency of A-r mutants of the recipient ($10^{-8.9}$). It seems likely that this strain had a mobilizable plasmid carrying A- and Ce-R.

The A-R in group 2 (A-r/Ca-s) could be mediated by a chromosomal penicillinase, a plasmid-mediated penicillinase and/or intrinsic resistance. Burman *et al.* (1968) compared two isogenic strains of *E. coli* K-12, one with a plasmid and one without. The former strain had a plasmid-mediated resistance with higher activity against A than the chromosomally mediated β -lactamase of the latter. A 20 μ g/ml was the highest concentration inhibiting growth for the strain without a plasmid against 100 μ g/ml A for the strain with a plasmid. The mean IC_{50} with A of group 2 (A-r/Ce-r) in this investigation was 81 μ g/ml and of group 3 (A-r/Ca-r) ≥ 1112 μ g/ml (Segaard 1979). The inoculum was much greater in the latter than in the former investigation ($10^{4.7}$ vs. $10^{1.0}$ CFU/ml). Chromosomally mediated resistance to A involves as the first step the *ampA* gene (Eriksson & Grennberg 1968). Higher resistance to A is obtained by addition of the *ampB* gene (Nordstrom *et al.* 1971). The sensitivity to Ca and Ce of *E. coli* strains with these genes was not recorded. The spontaneous mutants of the recipient and the group 2 strains in this paper might belong to such strains.

The strains in group 2 (A-r/Ca-s) were very sensitive to Ce β -lactamases of enzyme class I having high activity against cephalosporins.

Plasmid-mediated A-R in group 2 deserves some consideration. This plasmid could not be transferred in the direct assay but the A-R was inducible in some of the strains. Bobrowski *et al.* (1974) reported the finding of a plasmid-mediated β -lactamase indistinguishable from the chromosomal β -lactamase of *E. coli*. They mentioned the hypothesis that their plasmid originated from an *E. coli*

chromosome. This plasmid in an *E. coli* K-12 host gave resistance to A 32 μ g/ml, Ca 8 μ g/ml and Ce 125 μ g/ml (inoculum $10^{1.2}$ CFU). That was the same profile as the group 2 strains had in this investigation, and the resistance levels are about the same (A 11 μ g/ml, Ca 15 μ g/ml, Ce ≥ 421 μ g/ml, inoculum $10^{4.7}$ CFU/ml) (Segaard 1979). Thus it is possible that some of the group 2 bacteria harbour a mobilizable plasmid like that of Bobrowski *et al.* (1976).

Another explanation of the A-R observed in group 2 could be intrinsic resistance. In that case the outer membrane should be more permeable to Ca than to A and Ce. Plasmid-free *Pseudomonas aeruginosa* strains have the same profile. Some mutants of these strains have almost the same low MIC to A, Ca and cephalosporin (Richmond & Sykes 1973, Table 21). The resistance of the former strains is interpreted as intrinsic resistance, while the mutants have lost theirs.

There is also the possibility that the A-R of group 2 strains is based both on chromosomally-mediated penicillinase and intrinsic resistance as described by Boman *et al.* (1974).

The nature of the A-R in group 2 (A-r/Ca-s) can be further clarified by β -lactamase investigations.

I am indebted to Miss L.-L. B. Jørgensen for technical assistance.

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PURIFICATION AND CHARACTERIZATION OF ALEUTIAN DISEASE VIRUS

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Virus was isolated from infected mink organs by a combination of tissue homogenization fluorocarbon fraction and ultracentrifugation The final preparation was analysed by crossed immunoelectrophoresis and electron microscopy Virions had a capsid diameter of 22 nm Preparative agarose electrophoresis separated virions from contaminating ferritin Crossed immunoelectrophoresis of virus gave a single precipitate with sera from infected mink Crossed immunoelectrophoretic analysis with intermediate gels showed that a part of the virus preparation was complexed with antibody Serum from a certain mink was found to contain precipitating antibody to (poly)nucleotid Virus and virus antibody complexes were found to focus at pH 4.0-4.4 in isoelectric focusing In SDS polyacrylamide electrophoresis the main virus protein was found to have a molecular weight of 69000 This study gives further support to the classification of aleutian disease virus as a parvovirus

Key words Aleutian disease virus Plasmacytosis Parvo viridae

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Aleutian disease of mink (plasmacytosis) is caused by a persistent virus infection which gives a typical polyclonal hypergammaglobulinemia and complexes of virus antigen antibody and complement have been found in connection with glomerulonephritis and vasculitis in the terminal stage of the disease (16-17)

Most of the published physical-chemical parameters of aleutian disease (ADV) indicate that the virus is a parvovirus (13-16) These studies include the size and density of the particles (7) and the presence of virus-induced antigen(s) in the nucleus of infected cells (11) However the only two existing reports on peptide analyses of ADV agree about the presence of three low molecular weight polypeptides which are picorna virus like (15-20) and not characteristic for parvo-virus (19) Because of the possibility that some of the chemicals used in the standard purification procedure of ADV might be breakdown products of the virus polypeptide

we have chosen preparative electrophoresis as an alternative final purification method instead of density gradient ultracentrifugation which normally has been used (15-20) The intention of our studies has been to study the nature of the virus antigens by physical-chemical analyses and to characterize mink antibodies induced by the virus infection and thereby give an elucidation of the problem A preliminary report of the results has been given elsewhere (1)

MATERIAL AND METHODS

Infection of Animals

Sera from healthy mink were tested in counter-current electrophoresis (8) and found to give a negative reaction against ADV antigen These mink were then injected with organ material isolated from infected mink and killed on day 10 after infection The exact procedure has been described by Brummerstedt (5)

Production of Virus Antigen

Virus was partially purified by tissue homogenization in phosphate buffered saline (50 mM phosphate, 100 mM NaCl, pH 7.0) freezing and thawing the homogenate 4 times and fluorocarbon extraction (2 weight parts of homogenate mixed with 1 weight part of fluorocarbon) as described partly by Ingram & Cho (13). The supernatant was collected and the tissue phase was re-extracted with fluorocarbon. The combined supernatants were centrifuged at $7000 \times g$ for 45 min to remove remaining solids and the supernatant was ultracentrifuged at $156000 \times g$ for 2 h at 10°C . The pellet was re-extracted with fluorocarbon. (The step is also referred to as «activation» of virus, i.e. liberation of immunogenic determinants on virus).

Preparation of Mink Immunoglobulin

The virus antigen preparation was immobilized by coupling to N-hydroxysuccinimide activated Sepharose 4B as described by Cuatrecasas (9). 1 ml of this matrix was packed into a column and 1 ml of a pool of sera from 20 infected mink was passed through the column. The column was washed with 10 mM phosphate, 150 mM NaCl buffer pH 7.0 to A_{280} less than 0.01 and eluted with 0.2 M glycine buffer pH 3.0. The pH was finally adjusted to 7.0 with NaOH. The eluted protein was analysed by crossed immunoelectrophoresis (2) and sodium dodecyl sulphate polyacrylamide gelelectrophoresis (14) and found to be immunoglobulin.

Production of Rabbit Antisera

Rabbits were immunized subcutaneously with 100 μg of either the virus-antigen preparation or mink immunoglobulin preparation in Freund's incomplete adjuvant for antiserum preparation.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis with and without the use of intermediate gels was carried out as described by Axelsen *et al.* (2).

In some experiments Concanavalin A was used in the first dimension gel in a concentration of 100 μg per ml of agarose for glycoprotein identification (6).

Preparative Agarose Electrophoresis

Preparative agarose electrophoresis was carried out in 1% agarose blocks of $120 \times 100 \times 5$ mm in 20 mM barbital buffer pH 8.6. Electrophoresis time was about 2 h at 50 mA. After electrophoresis the agarose block was cut into 20 pieces ($120 \times 5 \times 5$ mm) and a small part of each piece ($5 \times 5 \times 5$ mm) was laid directly on an agarose gel containing serum from an infected mink or rabbit antiserum to the virus-antigen preparation (rocket electrophoresis see ref. 2). The agarose pieces positive for virus and ferritin (i.e. gave a precipitate in rocket electrophoresis) were centrifuged at $40000 \times g$ in 30 min at 4°C , and the supernatant was collected.

For isolation of the (poly)nucleotide present in the virus antigen preparation, preparative agarose electrophoresis with continuous elution was carried out (Savant preparative electrophoresis apparatus model PAG 15). The same buffer system as above was employed.

Isoelectric Focusing in Agarose Gels

Isoelectric focusing in agarose gels was carried out as described by Rosen *et al.* (18).

¹²⁵I Labelling

¹²⁵I labelling was carried out by the iodogen method (10), the chloramine method (12) or by the Bol Hunter method (4).

SDS-Polyacrylamidegel-Electrophoresis

SDS-polyacrylamidegel-electrophoresis was carried out in slab gels as described by Laemmli (14). Samples were heated to 100°C 1–2 min in sample buffer before electrophoresis.

Electronmicroscopy

Electronmicroscopy was performed by B. Bloch at department using uranyl acetate stain. The method has previously been described (5).

RESULTS

Purification of Aleutian Disease Virus

Crossed immunoelectrophoresis of virus antigen prepared by fluorocarbon extraction and different

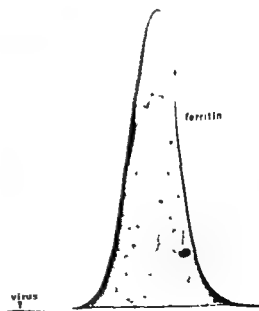


Fig. 1. Crossed immunoelectrophoresis of 10 μl of virus antigen preparation with 8.3 μl rabbit antiserum (11) of antigen preparation per cm^2 of second dimension agarosegel.

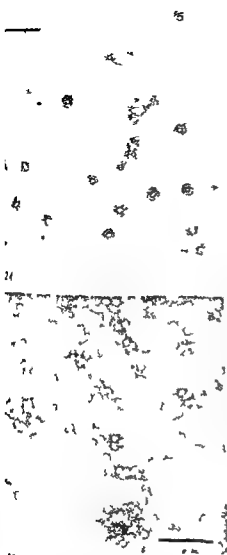


Fig. 2. Electron microscopy of virus (Fig. 2A) and ferritin (Fig. 2B) preparations isolated by preparative agarose electrophoresis. Magnification 140000 \times . The bar represents 100 nm.

centrifugation (see Material and Methods) is shown in Fig. 1 using a rabbit antiserum to the same preparation. Ferritin was the dominating protein as previously shown by electron microscopy (5). There are at least 100 times more ferritin molecules than virus particles. Ferritin and virus were separated by preparative agarose electrophoresis. The purity of the isolated ferritin and virus containing fractions was estimated by electron microscopy as shown in Fig. 2. No other structures were found in the virus preparation and vice versa.

Analysis of Virus by Crossed Immunoelectrophoresis

The virus preparation was analysed by crossed immunoelectrophoresis against both a rabbit antiserum to the virus antigen preparation (antiserum against both ferritin and virus) and sera from infected mink (antiserum to virus). Only a single precipitation arch was found in a position corresponding to the reaction between virus and antibody (Fig. 3).

When con A was incorporated into the first dimension agarose gel of a crossed immunoelectrophoresis no inhibition of electrophoretic migration was observed for virus. This indicates that no con A binding sugar residues are coupled to the virus capsid polypeptides.

Virus was radiolabelled with ^{125}I by three different iodination methods. No difference was found in iodination capacity of the different methods and generally only small amounts of iodine could be incorporated into the virus particles.

When radiolabelled virus was analysed by crossed immunoelectrophoresis exactly the same precipitation arch was found as the one shown in Figs. 1 and 3. Autoradiography of the electrophoresis is shown in Fig. 4. Only one precipitation arch was found, the one corresponding to the virus-antibody reaction. The form of this precipitation arch indicated that it consisted of a relatively strong symmetrical precipitation zone and a trail zone in the direction of the application well. This precipitate was analysed further in crossed immunoelectrophoresis with intermediate gels containing a rabbit antiserum to mink immunoglobulin(s) as shown in Fig. 5. It can be seen that the rabbit antibodies in the



Fig. 3. Crossed immunoelectrophoresis of 10 μl of virus preparation with 0.033 μl serum from an infected mink per cm^2 of second dimensions on agarose gel.

Fig 4 Autoradiography of virus antibody immune precipitate from crossed immunoelectrophoresis. The arrow indicates the application slit.

intermediate gel reacted only with the «trail zone» while the serum from the infected mink reacted mainly with the symmetrical (pure) virus component.

Estimation of the Isoelectric Point of Virus

Isoelectric focusing in agarose gels of virus followed by identification of virus by a second

pH Distribution

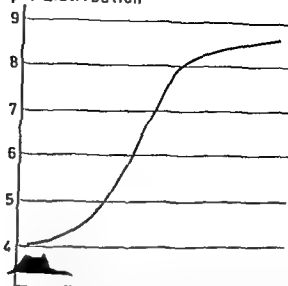


Fig 5 pH distribution in an isoelectric focusing agarose gel. After focusing the gel containing 50 μ l of virus was overlaid on a second dimension agarose gel containing 0.033 μ l serum from an infected mink per cm^2 of agarose gel for virus identification.

dimension electrophoresis of the focusing gel into an agarose gel containing antiserum to virus elucidated that virus (and virus antibody complexes) focused at pH 4.0–4.4. Three precipitation arches were seen by this technique as shown in Fig 6.

Demonstration of Free (Poly)Nucleotide in the Virus Antigen Preparations

A very fast moving molecule was found in certain virus antigen preparations when they were analysed in agarose electrophoresis. The molecule migrated to more than double the distance of the virus and the virus is in itself a fast moving particle. This fast moving molecule was isolated by preparative agarose electrophoresis. An absorption spectrum was obtained and the isolated molecule had a maximum absorbance at 260 nm. The

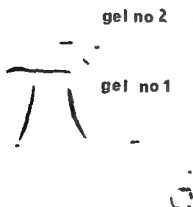


Fig 6 Crossed immunoelectrophoresis of 10 μ l of virus preparation with 10 μ l rabbit antiserum against mink immunoglobulin(s) per cm^2 of intermediate second dimension gel (gel no 1) and 0.03 μ l serum from an infected mink per cm^2 of second dimension gel (gel no 2).

Fig 7 Crossed immunoelectrophoresis of 10 μ l of virus antigen preparation with 0.033 μ l serum from mink no 24 per cm^2 of second dimension gel. The arrowhead indicates the application slit.

cule could be stained with toluidine blue and in
 use electrophoresis it migrated to exactly the
 distance as DNA preparation prepared from
 red pink organs. When serum from a certain
 (No 24) was incorporated into a second
 ion agarose gel precipitates were formed
 both the virus and the new molecule as
 in Fig 7.

Characterization of Virus Polypeptides

The virus preparation isolated from the prepara-
 tion agarose gel electrophoresis was analysed by
 polyacrylamide gel electrophoresis with and
 without 2 mercaptoethanol as seen in Fig 8.

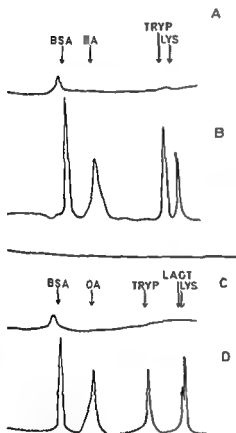


Fig 8 Densitometric scanning of an 11% SDS
 polyacrylamide gel of A 75 μ l of virus preparation
 B 5 μ l of 2 mercaptoethanol (2 ME) treatment
 C 4 marker proteins 5 μ g of each (BSA - Bovine
 serum Albumin of M_r 66000 OA - Ov Albumin of M_r
 14300 TRYP - Trypsinogen with M_r 24000 and LYS
 - Lysine with M_r 14300) without 2 ME treatment
 D 75 μ l of virus preparation with 2 ME treatment
 E 5 marker proteins 5 μ g of each (the same 4 as in B
 + 1 LACT - beta lactoglobulin with M_r 18400) with 2
 ME treatment

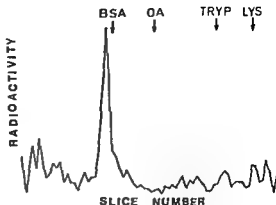


Fig 9 Radioactive distribution in an 11% SDS
 polyacrylamide gel of a 125 I labelled virus preparation

A single polypeptide with M_r 69000 was found.
 In one similar experiment a faint second band with
 M_r 59000 was also seen. 125 I labelled pure virus
 was also analysed by SDS polyacrylamide gel
 electrophoresis. The radioactive distribution in such
 a gel is shown in Fig 9 and it confirms the presence
 of a 69000 molecular weight band as did
 radioautography.

DISCUSSION

This study introduces a new electrophoretic method
 for virus purification. Normally ultracentrifugation
 in gradients has been used for the final virus
 purification step (7, 15, 20).

Virus was found to be partly covered by antibody
 giving a trail zone in crossed immunoelectrophoresis.

Virus (and virus antibody complexes) were found
 to focus at pH 4.0-4.4 in isoelectric focusing gels. It
 should be pointed out that there is an uncertainty in
 estimating isoelectric points at the end of a pH
 gradient as the one in Fig 11. However the pI values
 of 4.0-4.4 are consistent with the electrophoretic
 migration seen in normal agarose electrophoresis
 where virus migrated in front of human serum
 albumin used as a marker protein (pI of human
 serum albumin = 4.7). Because virus migration
 was not found to be inhibited by Con A incorpora-
 ted into the agarose gel during electrophoresis it is
 likely that virus capsid proteins are not glycosylated.
 Acid sugar residues coupled to virus proteins cannot
 therefore be responsible for the low isoelectric
 focusing point of virus.

Three precipitation arches were found when the
 isoelectric focused virus and virus antibody com-
 plexes were analysed by rocket electrophoresis. It is
 likely that the most acid particle (electrofocusing at

pH 4.0) is virus itself and the two other components are virus complexed with different ratios of antibody.

Free (poly)nucleotide was found in some virus antigen preparations. It is quite possible that the fluorocarbon extraction destroys some virus particles causing the liberation of virus (poly)nucleotide. The ultracentrifugation procedure used for the virus antigen preparation will pellet some of this (poly)nucleotide.

It was found that serum from a certain mink had antibody to this (poly)nucleotide. The production of such antibodies in infected mink has been reported before (3, 21). It is however uncertain how frequently sera from ADV infected and non ADV infected mink do contain such antibodies. The latter group of sera will most likely give false positive reactions in countercurrent electrophoresis for the diagnosis of ADV infections (8). This question is under study in our laboratory.

When various virus preparations were analysed by SDS polyacrylamide gel electrophoresis the main polypeptide was found to have a M_r of 69000. Occasionally a faint second band with M_r of 59000 was seen. When the same analyses were performed with ^{125}I labelled virus the presence of the M_r 69000 molecular weight component was confirmed. The 69000 and 59000 polypeptides found in this study of ADV are comparable to the polypeptides of the parvo viridae (19). The apparent inconsistency of the finding of these polypeptides with regard to the three low molecular weight polypeptides published by others (15, 20) cannot at present be explained but it may be suggested that the introduction of preparative agarose electrophoresis as the final purification method instead of gradient ultracentrifugation gave parvovirus related polypeptides when purified virus was analysed in SDS polyacrylamide gel electrophoresis.

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CHARACTERISTICS OF ANTISERA AGAINST PERIODATE-RESISTANT MEMBRANE ANTIGENS FROM *NEISSERIA GONORRHOEAE*

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Roe S F, Eggset G, Iversen O J & Mæland J A. Characteristics of antisera against periodate-resistant membrane antigens from *Neisseria gonorrhoeae*. Acta path microbiol scand Sect B 88 329-334 1980.

Crude outer membrane (OM) was prepared by extraction of bacteria of the *Neisseria gonorrhoeae* strains 8551 V and VII with an EDTA-containing buffer. The preparations contained the lipopolysaccharide (LPS) and at least 10 proteins as shown by SDS-polyacrylamide gel electrophoresis. Immunization of rabbits with untreated OM resulted in production of antibodies against several antigens including LPS. Antisera raised against periodate-treated OM did not contain antibodies against LPS. These latter antisera agglutinated heat-treated (100 °C, 60 min) gonococcal cells by means of antibodies to one or more common agglutinogens and against a strain-specific agglutinogen that was susceptible to digestion with proteolytic enzymes. Both slide agglutination and a plate agglutination test could be used to detect antibodies against these agglutinogens.

Key words: *Neisseria gonorrhoeae*, membrane antigens, periodate resistance.

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Gonococci are heterogeneous with regard to antigenic specificity of some of the surface structures of the bacterial cells. These structures include the cell wall of gonococci (3, 15) and components of the outer membrane (OM) such as the lipopolysaccharide (LPS) and OM proteins (4, 8, 9, 14). This knowledge has been attained by the use of different laboratory techniques, some of which may be too tedious for routine serotyping of the bacterium (16). Sandstrom & Danielsson (17) reported that when antisera to whole cells of gonococci were absorbed and used in a co-agglutination test, untreated gonococcal cells could be classified into three antigenic classes, each with a number of subtypes.

Results reported from this laboratory have shown that both OM proteins and LPS function as antigens in heated gonococci (1). Hence

serotyping of the bacteria on the basis of protein agglutinogens may be interfered with by anti-LPS antibodies and vice versa.

This report describes the results of experiments designed to raise antisera that contained antibodies to protein agglutinogens of the *Neisseria gonorrhoeae* strains 8551 V and VII but not antibodies to LPS. Rabbits were immunized with OM oxidized with periodate to destroy LPS and the antisera obtained were compared with sera from rabbits immunized with untreated OM.

MATERIAL AND METHODS

Bacterial Strains and Culture

The *N. gonorrhoeae* strains 8551 V and VII colony types T3 and T4 were cultured and harvested as previously (1) except that the culture medium used (18) was supplemented with the following antibiotics: Lincoc-

mycin chloride (1 µg/ml), Amphotericin B (1 µg/ml), Trimetoprim lactate (6.5 µg/ml), Colistin sodium methanosulphonate (6.5 µg/ml)

Preparation of Antigens

Mansheim & Kasper (10) The final preparations were suspended in distilled water, lyophilized, or kept frozen (8 mg/ml)

Lipopolysaccharide (LPS) LPS was prepared by extraction of whole gonococcal cells with phenol-water, and purified as reported earlier (12). To make it ready for the sensitization of sheep erythrocytes, lyophilized LPS was treated with NaOH and digested with pronase as reported (14).

Antigenic determinant b A crude preparation was prepared by the extraction of whole gonococcal cells with alkali (11). The preparation was treated with periodate to destroy LPS and was then used for the sensitization of sheep erythrocytes with the protein determinant *b* as described previously (12).

Serological Methods

Bacterial agglutination The titre of antiserum against heat-treated gonococci (100 °C 60 min) was determined using a glass plate agglutination test as reported (1). Slide agglutination testing was performed by the mixing of one drop of antiserum diluted in phosphate buffered saline pH 7.2 (PBS) with one drop of the suspension of heated gonococci standardized to a turbidity of a McFarland No. 5 standard. The slide was agitated by hand for up to 150 s and the agglutination read in oblique light was graded as follows: agglutination appearing within 30 s (3+) agglutination appearing from 30 to 90 s (2+) agglutination appearing from 90 to 150 s (1+) no agglutination after 150 s (-).

Indirect haemagglutination Sheep erythrocytes were sensitized with LPS or the *b* determinant of gonococci as described earlier (12). The assay was performed using a microtitre equipment and the titres of antiserum determined as described (6).

Immunodiffusion Double diffusion slides were prepared and the test was performed as described previously (2). The wells were filled with undiluted antiserum or with OM (4 mg/ml) dissolved in an endotoxin-disaggregating buffer (10) that contained Na-deoxycholate (NaD buffer). The gels were stained for proteins with Coomassie brilliant blue (21).

Other Methods

Immunization Rabbits were immunized with untreated or periodate-oxidized OM preparations suspended in 0.85 per cent NaCl. The animals received 0.5 mg of the preparations intramuscularly twice each week for a period of three weeks and then 1 mg doses twice in the sixth and twice in the seventh week of the immunization period. The animals were bled 7 days after the last injection. The antisera obtained, anti-OM from animals receiving untreated OM, and anti-POM from those

injected with periodate-treated OM, were stored at -20 °C, and heated at 56 °C for 30 min before testing.

Absorption of antiserum Absorption with untreated or heat treated gonococci was performed as previously described (1). For the absorption, 2 g of wet bacteria was used per 0.1 ml of undiluted antiserum. The same amount of antiserum was absorbed with 4 mg of the OM preparation with incubation and centrifugation as reported (1).

of the OM preparation in 0.7 ml volume of the same buffer was applied. The gradient was centrifuged (Beckman Model LS-65) at 105000 × *g* for 18 h. Fractions of 0.6 ml each were collected by a density gradient fractionator (Isco, Model 640). Then 0.1 ml of each fraction was mixed with 0.9 ml of 0.1N NaOH and the optical density was measured at 280 nm.

Electron microscopy OM in distilled water (0.25 mg/ml) was prepared and stained with 2 per cent (w/v) sodium phosphotungstate, pH 6.6 for 30 s (5). The preparations were examined by electron microscopy (Jeol JEM-100 CX).

SDS-polyacrylamide gel electrophoresis Electrophoresis was performed essentially as described by Weber & Osborn (20) using the protein standards buffers and staining methods reported earlier (6).

Periodate oxidation The OM preparation was suspended in 0.5 per cent Na meta-periodate (Merck) in distilled water (1 mg/ml) kept at 4 °C for 20 h, dialyzed against water for 24 h and then against saline. The preparations were kept frozen and were thawed immediately before being used for immunization.

Enzyme digestion All the enzyme preparations were manufactured by Calbiochem. Digestion with trypsin or pronase was performed using a 0.05M Tris HCl buffer, pH 7.5 with pepsin using a 0.05M citrate phosphate buffer pH 2.6 and with papain using a 0.1M phosphate buffer pH 7.4 with the addition of 0.01M cysteine and 0.002M EDTA. The digestion was performed at 37 °C for 4 h with a substrate to enzyme ratio of 16:1 (w/w). The enzyme activity was destroyed by heating the preparation at 100 °C for 5 min. The mixtures were then dialyzed against water lyophilized and used for the absorption of antiserum as described above. An undigested control preparation was included for each of the enzymes.

RESULTS

Electron microscopy of the OM preparations showed material consisting of aggregates of membranous structures of variable shape, most of them bounded by a dense layer (Fig. 1). On sucrose gradient centrifugation a single band between 1.18 and 1.23 g/ml of sucrose was observed (Fig. 2). SDS-polyacrylamide gel electrophoresis showed that each preparation contained at least 10 different proteins, some of which were present in very low



Fig 1 Electron micrograph of an outer membrane vesicle from the *N gonorrhoeae* strain VIII, stained with sodium phosphotungstate pH 7.0 ($\times 90,000$)

concentrations (Fig 3). The preparations contained a precipitating protein with a molecular weight of 900 daltons for each of the strains 8551 and VII and 38000 daltons for strain V. Staining for carbohydrates with Alcian blue showed a faint orange line in all three preparations with a R_f corresponding to that of cytochrome C (300 daltons).

Double diffusion in agar gel analysis of the OM preparations against the corresponding anti OM or anti pOM sera showed similar patterns of precipitation lines with all three strains exemplified in Fig 4. Both antisera gave rise to a precipitation line close to the antigen basin and one line midway between the antigen and serum basins. The anti OM but not the anti pOM serum also showed a line close to the serum basin. This pattern of precipitation lines was not observed when OM was dissolved in the NaD buffer. No precipitation was observed when the preparations were suspended in PBS and tested.

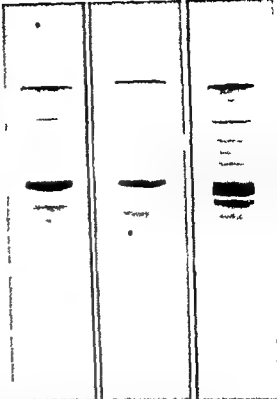


Fig 3 SDS polyacrylamide gel electrophoresis of the outer membrane from the *N gonorrhoeae* strains 8551 (left), V (middle) and VII (right).

Indirect Haemagglutination Testing

The anti OM sera contained antibodies both against LPS and the protein determinant *b* of the corresponding strains (Table 1). Antibody activity against determinant *b* was also shown by the anti pOM sera but they showed no antibody activity against LPS.

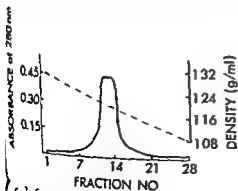


Fig 4 Sucrose gradient centrifugation of the outer membrane from the *N gonorrhoeae* strain VII. Solid line shows absorbance at 280 nm and broken line density of sucrose.



Fig 4 Double diffusion in agar gel with anti-OM 8551 (lower left) and anti-pOM 8551 serum (lower right) against outer membrane from the *N gonorrhoeae* strain 8551 (upper well).

mycin chloride (1 µg/ml), Amphotericin B (1 µg/ml), Trimetoprim lactate (6.5 µg/ml), Colistin sodium methanesulphonate (6.5 µg/ml)

Preparation of Antigens

Outer membrane Extraction of whole gonococcal cells with an EDTA containing buffer and purification of the OM by centrifugation were performed as described by Mansheim & Kasper (10). The final preparations were suspended in distilled water, lyophilized, or kept frozen (8 mg/ml).

Lipopolysaccharide (LPS) LPS was prepared by extraction of whole gonococcal cells with phenol-water, and purified as reported earlier (12). To make it ready for the sensitization of sheep erythrocytes, lyophilized LPS was treated with NaOH and digested with pronase as reported (14).

Antigenic determinant b A crude preparation was prepared by the extraction of whole gonococcal cells with alkali (11). The preparation was treated with periodate to destroy LPS and was then used for the sensitization of sheep erythrocytes with the protein determinant *b* as described previously (12).

Serological Methods

Bacterial agglutination The titre of antiserum against heat treated gonococci (100 °C, 60 min) was determined using a glass plate agglutination test as reported (1). Slide agglutination testing was performed by the mixing of one drop of antiserum diluted in phosphate buffered saline pH 7.2 (PBS) with one drop of the suspension of heated gonococci standardized to a turbidity of a McFarland No. 5 standard. The slide was agitated by hand for up to 150 s.

Agglutination was graded as follows:
30 s (3+)
0 s (2+)
1 s (1+) no agglutination after 150 s (-)

Indirect haemagglutination Sheep erythrocytes were sensitized with LPS or the *b* determinant of gonococci as described earlier (12). The assay was performed using a microtitre equipment and the titres of antiserum determined as described (6).

Immunodiffusion Double diffusion slides were prepared and the test was performed as described previously (2). The wells were filled with undiluted antiserum or with OM (4 mg/ml) dissolved in an endotoxin disaggregating buffer (10) that contained Na-deoxycholate (NaD buffer). The gels were stained for proteins with Coomassie brilliant blue (21).

Other Methods

Immunization Rabbits were immunized with untreated or periodate oxidized OM preparations suspended in 0.85 per cent NaCl. The animals received 0.5 mg of the preparations intramuscularly twice each week for a period of three weeks and then 1 mg doses twice in the sixth and twice in the seventh week of the immunization period. The animals were bled 7 days after the last injection. The antisera obtained anti-OM from animals receiving untreated OM and anti-pOM from those

injected with periodate-treated OM were stored at -20 °C, and heated at 56 °C for 30 min before testing.

Absorption of antiserum Absorption with untreated, heat treated gonococci was performed as previous described (1). For the absorption 0.2 g of wet bacteria was used per 0.1 ml of undiluted antiserum. This same amount of antiserum was absorbed with 4 mg of the OM preparation with incubation and centrifugation as reported (1).

Sucrose gradient centrifugation A linear gradient of sucrose, from 20 to 65 per cent (w/w) in a total volume of 16 ml was prepared in the EDTA buffer (10) and 7 ml of the OM preparation in 0.7 ml volume of the same buffer was applied. The gradient was centrifuged (Beckman Model L5-65) at 105000 × *g* for 18 h. Fractions of 0.6 ml each were collected by a density gradient fractionator (Isco, Model 640). Then 0.1 ml of each fraction was mixed with 0.9 ml of 0.1N NaOH and the optical density was measured at 280 nm.

Electron microscopy OM in distilled water (0.25 mg/ml) was prepared and stained with 2 per cent (w/v) sodium phosphotungstate pH 6.6 for 30 min (3). The preparations were examined by electron microscopy (Jeol JEM-100 CX).

SDS-polyacrylamide gel electrophoresis Electrophoresis was performed essentially as described by Weber & Osborn (20), using the protein standards buffers and staining methods reported earlier (6).

Periodate oxidation The OM preparation was suspended in 0.5 per cent Na-meta-periodate (Merck) in distilled water (1 mg/ml) kept at 4 °C for 20 h, dialyzed against water for 24 h, and then against saline. The preparations were kept frozen and were thawed immediately before being used for immunization.

Enzyme digestion All the enzyme preparations were manufactured by Calbiochem. Digestion with trypsin or pronase was performed using a 0.05M Tris HCl buffer pH 7.5 with pepsin using a 0.05M citrate phosphate buffer pH 2.6 and with papain using a 0.1M phosphate buffer pH 7.4 with the addition of 0.01M cysteine and 0.002M EDTA. The digestion was performed at 37 °C for 4 h with a substrate to enzyme ratio of 16:1 (w/w). The enzyme activity was destroyed by heating the preparation at 100 °C for 5 min. The mixtures were then dialyzed against water, lyophilized and used for the absorption of antiserum as described above. An undigested control preparation was included for each of the enzymes.

RESULTS

Electron microscopy of the OM preparations showed material consisting of aggregates of membranous structures of variable shape, most of them bounded by a dense layer (Fig. 1). On sucrose gradient centrifugation a single band between 1.18 and 1.23 g/ml of sucrose was observed (Fig. 2).



Fig 1 Electron micrograph of an outer membrane preparation from the *N gonorrhoeae* strain VIII negatively stained with sodium phosphotungstate pH 5 ($\times 90\,000$)

preparations (Fig 3). The preparations contained dominating protein with a molecular weight of 9900 daltons for each of the strains 8551 and VII and 18000 daltons for strain V. Staining for carbohydrates with Alcian blue showed a fast moving line in all three preparations with a mobility corresponding to that of cytochrome C (1500 daltons).

Double diffusion in agar gel analysis of the OM preparations against the corresponding anti-OM or anti-pOM sera showed similar patterns of precipitation lines with all three strains exemplified in Fig 4. Both antisera gave rise to a precipitation line close to the antigen basin and one line midway between antigen and serum basins. The anti-OM but not anti-pOM serum also showed a line close to the serum basin. This pattern of precipitation lines was observed when OM was dissolved in the NaD buffer. No precipitation was observed when the preparations were suspended in PBS and tested.

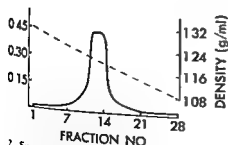


Fig 2 Sucrose gradient centrifugation of the outer membrane from the *N gonorrhoeae* strain VII. Solid line shows absorbance at 280 nm and broken line density.

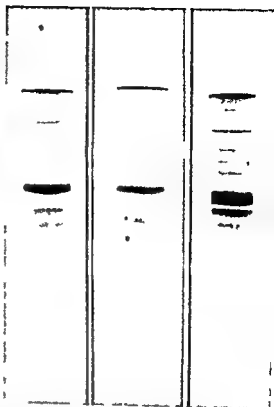


Fig 3 SDS polyacrylamide gel electrophoresis of the outer membrane from the *N gonorrhoeae* strains 8551 (left), V (middle) and VII (right).

Indirect Haemagglutination Testing

The anti-OM sera contained antibodies both against LPS and the protein determinant *b* of the corresponding strains (Table 1). Antibody activity against determinant *b* was also shown by the anti-pOM sera but they showed no antibody activity against LPS.



Fig 4 Double diffusion in agar gel with anti-OM 8551 (lower left) and anti-pOM 8551 serum (lower right) against outer membrane from the *N gonorrhoeae* strain 8551 (upper well).

TABLE 1 *Titres of Anti OM 8551 OM V and OM VII Sera and the Corresponding Anti pOM Sera Against Erythrocytes Sensitized with LPS or Determinant b of the Homologous N gonorrhoeae Strains*

Antiserum	Antigen	
	LPS	Det b
Anti OM 8551	256	512
Anti pOM 8551	<4	1024
Anti OM V	256	1024
Anti pOM V	<4	1024
Anti OM VII	256	2048
Anti pOM VII	<4	2048

LPS was oxidized with periodate and then used for the sensitization of erythrocytes. Antibody agglutinating the erythrocytes was not present in any of the antisera examined.

Antibody to the *b* determinant was removed by absorption of the antiserum with untreated or heated gonococci, no matter whether homologous or heterologous bacteria were used. Antibody activity to LPS in absorbed or cross absorbed antisera was not studied.

Bacterial Agglutination with Anti pOM Serum

The antisera strongly agglutinated the homologous or heterologous heated gonococci in slide agglutination testing and in the plate agglutination test with the titres shown in Table 2. On absorption of any of the antisera with one or both of the heterologous strains only the homologous strain was agglutinated. The agglutinating capacity of the antisera after cross absorption with both of the heterologous strains is shown in Table 3. This antibody activity could be eliminated by absorption with the homologous gonococci or OM preparation.

When the antisera were tested against unheated gonococci, only weak agglutination was observed and the formation of the agglutinates was delayed in comparison with that observed with the heat treated bacteria.

TABLE 2 *Titre in Bacterial Agglutination with Anti pOM 8551 pOM V and pOM VII Sera*

Antiserum	Antigen		
	8551	V	VII
Anti pOM 8551	1024	512	512
Anti pOM V	128	256	256
Anti pOM VII	512	512	1024

TABLE 3 *Slide and Plate Agglutination with Cross Absorbed Anti pOM Sera Against the N gonorrhoeae Strains 8551 V and VII*

Antiserum	Antigen		
	8551	V	VII
Anti pOM 8551	3+(128) ^a	-(<4)	-(<4)
Anti pOM V	-(<4)	2+(64)	-(<4)
Anti pOM VII	-(<4)	-(<4)	3+(256)

^a) Denotes intensity of reaction in the slide agglutination test with antiserum diluted 1:4 and in the plate agglutination test.

The agglutination experiments were also performed with gonococci grown without antibiotic agents added to the culture medium. Agglutination similar to that described was obtained with the bacteria.

Enzyme Digestion

Each of the OM preparations was digested with the various proteolytic enzymes. The final lyophilized preparation was then used for the absorption of a portion of the corresponding anti pOM serum that had already been absorbed with the heterologous bacteria (see Table 3). Digestion with pronase best destroyed the absorption destroyed the ability of all three OM preparations to remove the antibodies agglutinating the corresponding gonococcal cells (Table 4). Pepsin digestion had a similar effect on the OM preparations from the strains 8551 and VII but not the strain preparation. Papain or trypsin digestion did not destroy the antibody absorbing ability of the preparations. The results demonstrate susceptibility of the OM antigen that functioned as agglutinin in tests with the cross absorbed anti pOM sera.

Treatment of the OM preparations with periodate did not affect their antibody-combining ability.

TABLE 4 *Slide Agglutination with Cross Absorbed Anti pOM Sera Against the Homologous Gonococcus after Absorption of Antisera with Untreated or Enzyme Digested Outer Membrane*

OM digested with	Anti pOM		
	8551	V	VII
Papain	-	-	-
Pepsin	2+	-	2+
Pronase	3+	2+	2+
Trypsin	-	-	-
None	-	-	-

DISCUSSION

materials prepared from the *N. gonorrhoeae* with the EDTA buffer were greatly enriched in membrane structures that showed banding as a peak at a density of from 1.18 to 1.23 g/ml in a gradient centrifuged at 105000 \times g for 2 h.

These findings strongly indicate that the outer membrane protein of gonococci (7, 8) was greatly enriched in the outer membrane of the bacteria. However, the possibility that the OMI preparations were contaminated with proteins of a different origin, such as cytoplasmic membrane proteins, can not be excluded. The gel precipitation showed that the anti-OMI contained antibodies against at least three different precipitinogens and the anti-pOMI sera contained at least two of them. Both categories of antibodies in the protein determinations are probably produced by all gonococci and meningococci (13). Anti-OMI but not anti-pOMI contained anti-LPS antibodies. This finding with earlier observations showing that the treatment destroyed the antibody-combining sites of gonococcal LPS (12). Nor was the production against periodate-oxidized LPS inhibited in spite of the earlier observations that the carbohydrate moiety of this component was completely disintegrated by the treatment (14). These results strongly support the notion that the reaction of antibodies to LPS can be avoided by treatment of the antigen with periodate prior to immunization and that this treatment does not interfere with antibody production against periodate-resistant antigens.

The anti-pOMI sera agglutinated the heat-treated gonococci in accordance with earlier observations indicating that surface proteins function as agglutinins in the gonococcus (1). The periodate-resistant agglutinin(s) common to the strains 8551 V and VII and a seemingly strain-specific agglutinin. That this latter agglutinin is of protein nature was substantiated by its sensitivity to pronase and for the strains 8551 V and VII to pepsin as well.

Ström & Danielsson (17) recently reported results from a study of gonococcal

serotypes detected utilizing cross-absorbed antisera in a coagglutination test. They divided the gonococci into the antigen classes W, J and M. The heat-resistant W class antigens were mostly of protein nature and were subdivided into the groups W/I, W/II and W/III. They showed that the strains V, II and V used in this study belonged to the group W/I and W/II respectively and that strain 8551 reacted with the W/III as well as a W/II antibody reagent. The strain-specific antigens detected in the present study by direct agglutination of the bacteria may correspond to the group antigens of the W antigen class of gonococci. Also, the OMI proteins characterizing the strains V, II and 8551 may correspond to the immunotypes A, B and C detected by Wang *et al.* (19) using mouse antisera to gonococci in a micro-immunofluorescence test. The present demonstration of serological diversity of proteins in gonococci is also consistent with results reported by other investigators (4, 8, 9) although it seems less clear how these results correspond to those of the present study.

The experiments described were performed with gonococci grown in the presence of antibacterial agents to eliminate the growth of contaminating bacteria. However, when gonococci cultured without antibiotics in the medium were used as antigen, they showed the same results in the bacterial agglutination tests, indicating that neither the common nor the strain-specific antigen was affected by the agents used.

In studies not yet completed, we have found that the majority of gonococcal isolates can be readily classified by slide agglutination using the cross-absorbed anti-pOMI sera described in this study.

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ANTIBODIES TO THE STRAIN-SPECIFIC AND CROSS-REACTIVE DETERMINANTS OF THE HAEMAGGLUTININ OF INFLUENZA H3N2 VIRUSES

2 Antiviral Activities of the Antibodies in Biological Systems

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Haaheim L. R. & Schild G. C. Antibodies to the strain specific and cross reactive determinants of the haemagglutinin of influenza H3N2 viruses. 2. Antiviral activities of the antibodies in biological systems. Acta path microbiol scand Sect B 88 335-340 1980

Antibodies to the strain specific (SS) and cross reactive (CR) antigenic determinants of the haemagglutinin (HA) of influenza H3N2 viruses were prepared and their antiviral properties in experimentally infected mice and in eggs were investigated. The SS antibodies were considerably more efficient than the CR antibodies in direct virus neutralization tests. The difference in the activities of the two types of antibody preparations was less pronounced in virus growth inhibition studies in the allantois-on-shell system and in passive protection studies in mice given antibody before challenge with virus. Nevertheless, a more effective than was CR antibody preparations virus on day three

was observed compared with the control animals. The degree of reduction in titre exerted by the SS antibodies was greater than for the CR antibodies. In contrast the rate of clearance of pulmonary virus 9-12 days after infection in the mice treated with SS antibody was slower than for the CR treated animals. Also in SS antibody treated animals viruses isolated from mouse lungs showed evidence of minor antigenic variation away from the parent virus.

Key words: Influenza, haemagglutinin, strain specific antibodies, cross reactive antibodies, biological activity.

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Antibody to the haemagglutinin (HA) antigen of influenza virus is considered to be an important factor in protection against infection (see reviews 8, 9). The different antigenic determinants of the HA molecule of influenza A viruses can operationally be divided into the strain specific (SS) determinants which undergo frequent minor changes (antigenic drift), and the cross reactive (CR) determinants which are less antigenically common

for a range of viruses within a subtype (6, 7, 12).

We have previously shown that the serological potencies in single radial-diffusion tests and haemagglutination inhibition assays for antibodies to the SS and CR antigenic determinants differ markedly. Antibodies to the SS determinants exhibited higher *in vitro* serological activity per µg IgG than did antibodies to the CR determinants, a finding that could be due to the presence of more CR than SS determinants on the HA molecule (6).

The present report describes the anti viral activities of these antibodies in biological systems. The activities of antibody preparations against the SS and CR determinants were investigated in virus neutralization tests in virus growth inhibition experiments in the allantois on shell (AOS) system and in challenge studies in mice passively given antibody before infection.

MATERIALS AND METHODS

Virus Strains Immune Sera Strain specific and Cross reactive Antibody Preparations

All test strains sera and antibody preparations were as described previously by Haasheim & Schild (6). In brief immune goat serum to purified HA antigen from A/Hong Kong/1/68 (H3N2) virus was exhaustively absorbed with strains of the H3N2 subtype namely A/England/42/72 and A/Victoria/3/75 in order to obtain different preparations of strain specific anti HK/68 HA antibodies (SS and SS respectively). The cross reactive antibodies (CR and CR respectively) were isolated by eluting antibodies from the absorbing viruses. A control goat serum free of anti H3 antibodies was prepared by the complete absorption of antiserum with HK/68 Eng/72 and Vic/75 viruses and subjected to DEAE cellulose chromatography in order to obtain a pure IgG preparation. This material is referred to as Non HA IgG.

HK/68 virus was adapted to become lethal to mice by five consecutive skim passages in mice according to Faekas de St Groth & Tees (3).

Mice

Adult outbred female mice (NMRI/Bom) weighing between 20–22 g were used.

Antibody Assays

Virus neutralization (VN) tests These were performed by mixing serial 10 fold dilutions of antibody preparations with 100 egg infective doses (50%) (EID₅₀) and incubating the mixtures for 30 min at room temperature before inoculating allantoically into 6 eggs per antibody dilution. The 50% virus neutralization end point was calculated (9).

Virus neutralization in mice was performed by mixing equal volumes of serial 10 fold dilutions of antibody and 4 mouse lethal doses (50%) (MLD₅₀) of mouse adapted HK/68 virus. The mixtures were incubated for 30 min room temperature and then kept at 0 °C while performing intranasal inoculation into mice. Five anaesthetized animals were given 50 µl of each antibody virus mixture. Mortality was recorded and the experiments were terminated 10 days post infection.

Growth inhibition studies Cultures of fragments of chorio allantoic membranes attached to the egg shell (AOS cultures) were prepared as described by Faekas de St Groth & White (2). For growth inhibition tests serial 3–16 fold dilutions (1/2 log₁₀ steps) of antibody were made and each AOS culture was challenged with 100 AOS₅₀ doses of virus (5).

In the mouse system groups of 5 animals were given 0.2 ml volumes of each antibody dilution intraperitoneally one day before challenge with 4 MLD₅₀ of mouse adapted HK/68 virus and mortality was recorded for 14 days post infection.

In two independent studies groups of mice were given different goat IgG antibody preparations (2 µg IgG animal) or PBS one day before intranasal challenge with 0.1 MLD₅₀ of virus. Three to four animals were killed on days 3, 6, 9 and 12 post infection and the remaining animals were killed 20 days (Experiment 1) or

TABLE 1. Direct Virus Neutralization Properties of SS and CR Antibody Preparations Obtained from Immune Goat Serum to Purified HA Antigen of A/Hong Kong/1/68 (H3N2) Virus

Antibody preparation tested ^{a)}	Indicator system for virus infectivity		Mice	
	Embryonated eggs			
	Log ₁₀ titre/ml	Spec act ^{b)}	Log ₁₀ titre/ml	Spec act
SS	4.5	80	4.5	80
CR	2.9	4	3.4	12
SS	4.7	280	4.8	350
CR	4.2	30	4.4	45
Non HA IgG	<1.5	<1	ND ^{c)}	

^{a)} SS (SS⁺) represents residual (strain specific) antibodies after absorption with Vic/75 (Eng/72) virus whereas CR (CR⁺) represents cross reactive antibodies eluted from the absorbing strain. Non HA IgG is IgG fraction without anti influenza HA activity.

^{b)} Specific activity presented as ratio of geometric mean titre of duplicate tests to IgG concentration of antibody (µg/ml).

^{c)} Not done.

TABLE 2. Growth Inhibition Tests with SS and CR Antibody Preparations Obtained from Immune Goat Serum to Purified HA Antigen of A/Hong Kong/168 (H3N2) Virus

AOS inhibition tests ⁽¹⁾					Passive protection studies in mice (representative experiment)							
SS	CR	SS	CR	Non HA IgG	SS		CR		SS		CR	
					µg IgG/ animal	death rate	µg IgG/ animal	death rate	µg IgG/ animal	death rate	µg IgG/ animal	death rate
Log in titre (duplicate tests)					39.8	0/5 ⁽²⁾	21.3	0/5	18.3	0/5	16.9	0/5
1	3.4	3.8	4.0	<1.0	12.6	1/5	6.7	1/5	5.8	0/5	5.4	0/5
					4.0	0/5	2.1	3/5	1.8	1/5	1.7	1/5
Specific activity (titre/µg IgG)					1.3	1/5	0.7	5/5	0.6	2/5	0.5	2/5
					0.4	5/5	0.2	5/5	0.2	5/5	0.2	4/5
II	12	36	20	<1	IgG mouse protective dose (50%) MPD ₅₀ for duplicate tests							
					D 9		4.0		1.0		1.0	

¹ Allantoin-on shell inhibition tests see Materials & Methods

² Ratio of cumulative number of dead mice at day 10 post infection to number of test animals in the group given antibody intraperitoneally one day before challenge with 4 MLD₅₀ of HK/68 virus

in haemagglutination inhibition (HI) antibodies against strains HK/68, Eng/72 and Vic/75 by standard methods (16)

RESULTS

Direct Virus Neutralization Activity

Dilutions of antibody preparations were incubated with standard concentrations of virus (100 ID₅₀ or 4 MLD₅₀) before inoculating into embryonated eggs or given intranasally to mice. The results of these experiments are presented in Table 1. All antibody preparations showed evidence of neutralizing virus infectivity but there were marked differences in their specific activities (titre/µg IgG). The most active preparation was the narrowly reactive SS antibody (SS) whereas the most broadly reactive CR antibody (CR) showed the least specific activity. The relative activities were similar to those found previously for *in vitro* assays (6).

Virus Growth Inhibition Activity

AOS inhibition tests These experiments were done in the AOS system where pieces of chorionic membrane attached to egg shell were infected with 100 AOS₅₀ doses of virus and maintained in medium containing dilutions of antibody. The results of these assays are presented in Table 2. The difference between the specific activities of the various antibody preparations tested was smaller than in the neutralization experiments

described above. Even the most broadly reacting CR antibody (CR) performed well in this type of assay.

Passive mouse protection tests Groups of mice

conferred passive protection against fatal infection. The experiments were performed in duplicate and the minimum protective dose of IgG necessary to inhibit death in 50% of the animals was calculated. As for the AOS inhibition tests, the 50% protective dose for these antibody preparations fell within a limited range, the SS antibodies being about 4 fold more active per quantity of IgG than the CR antibodies.

Effect of Passively Given Anti-HA Antibody on a Sublethal Murine Influenza Infection

Effect on pulmonary virus In order to investigate whether viral replication in mouse lungs was modified by the presence of non protective levels of passively administered anti HA antibody before infection, animals were given 2 µg IgG of either SS, CR or Non HA IgG, others were given PBS only before challenge with 0.1 MLD₅₀ of HK/68 virus (Experiment 1). In a duplicate test (Experiment 2) PBS was employed as a placebo. The results of both experiments (Fig. 1) were similar. Firstly, the greatest reduction of pulmonary virus titre on day 3 post infection was observed for mice treated with SS antibody. Secondly, both experiments showed that at day 9 post infection the

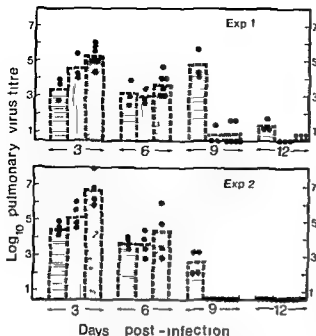


Fig 1 Geometric mean titres of pulmonary virus assayed in the AOS system in groups of mice given 2 µg IgG of SS antibody (horizontally shaded histograms) or CR antibody (vertically shaded histograms) intraperitoneally 1 day before challenge with 0.1 MLD₅₀ of HK/68 virus. The darker shaded histograms represent the placebo treated animals. In experiment 1 (upper panel) the placebo group consists of animals given either PBS or 2 µg of Non HA IgG whereas in experiment 2 (lower panel) only PBS was employed as a control. Individual titres are presented as black dots. Titres < 1 (log₁₀ titre < 1) located on or just above the abscissa are assigned the value of 5 when calculating mean titres.

clearance of virus from the lungs were delayed in animals treated with SS antibody.

Viruses isolated on day 9 post infection were assayed in HI tests with the original goat serum, purified HK/68 HA antigen and the two antibody subfractions SS and CR (Table 3). Viruses isolated from the SS treated animals showed evidence of minor antigenic drift away from the parent HK/68 virus when tested with SS antibody (> four fold reduction in HI titres compared with the parent virus) and from the viruses isolated from the CR treated and placebo treated animals. HI tests with anti HK/68 HA serum or with CR antibody did not reveal evidence of antigenic differences between the strains.

Effect on post infection HI antibodies. It has been observed in man (10, 14) that inactivated influenza vaccines administered to primed individuals possessing prevaccination CR antibody to H3N2 virus rarely evoke SS antibodies to the vaccine virus. A possible explanation for these observations is that the CR antibody may have a blocking effect on the expression of SS determinants. It was therefore of interest to see whether passively administered CR or SS antibody interfered with the anti HA antibody response in experimentally infected mice. Our experiments described above revealed no effect of this kind. Sera collected from the various treatment groups of mice on day 20 or 28 post infection all had comparable levels of post infection HI antibodies to both the homologous HK/68 virus and the heterologous H3N2 variants Eng/72 and Vic/71 (data not shown). Control mice given antibody but not infected had low or undetectable levels of serum HI activity throughout the experiment. Thus it appears that the amount of passively administered

TABLE 3 HI Tests with Pulmonary Virus Isolated on Day 9 Post Infection with A/Hong Kong/1/68 (H3N2) Virus and Coat Antiserum to Purified HA Antigen of HK/68 Virus and Two Antibody Subfractions CR and SS

Mice treated with	Virus isolates tested	Antibody preparations		
		HK/68 goat serum	CR	SS
SS antibody	511	12.0 ^a	7.1	6.3
	512	11.3	7.3	6.1
	530	12.8	8.1	7.6
CR antibody	531	13.3	7.6	8.6
	532	13.0	7.4	8.3
	552	13.1	7.6	7.9
PBS (control)	553	12.8	7.4	8.3
	554	13.3	7.9	7.8
	574	13.6	8.4	8.1
	A/HK/1/68	13.1	7.9	8.6

^a Log₂ HI titre of geometric mean of duplicate tests

CR antibody given in mice in our experiments, a measureable effect on the expression of the S antigenic determinants on the HA molecule infecting virus.

DISCUSSION

We have previously found that the *in vitro* properties of SS and CR anti HK/68 antibodies obtained from immune goat serum differed markedly. The most narrowly reacting SS (SSⁿ) exhibited higher specific activity per μ g of IgG than did the most broadly reacting antibody (CR) (6). Our observations have now extended to include their antiviral properties including direct virus neutralization, passive protection of mice against lethal challenge, inhibition of virus growth in AOS cultures and effect on virus growth in mice after a sublethal challenge.

In the present studies using direct VN tests and exposure of virus to antibody *in vitro* by using embryonated eggs or susceptible mice as host systems for virus infectivity, considerable differences were detected in their specific activities. SSⁿ antibodies being about 30-70 fold more active than the CR antibodies. This difference in specific activity was greater than that detected between these preparations in HI tests (6). However, the difference in specific activities of the antibody was less pronounced in virus growth inhibition tests in AOS system or in passive protection of mice against lethal challenge. Nevertheless, even in these systems the SS antibodies were somewhat more effective (3-4 fold) than the CR antibodies. The results of our mouse protection studies using intraperitoneally administered antibodies were in accordance with those of Virelizier (11) who used influenza A viruses of the H₃N₂ H1N1 subtype. He observed better protection by strain specific HA antibodies than by the cross reactive bodies.

These assays and passive mouse protection assays are of obvious relevance to our understanding of the biological parameters of immunity to influenza. In AOS inhibition tests and titration of pulmonary virus in infected mice are biologically relevant in that they provide information on the ability of antibody to modify viral replication once infection is established. When mice were passively immunized with small quantities of narrowly reacting strain specific (SSⁿ) anti HA antibody before challenge with a sublethal dose of HK/68 virus pulmonary virus titres were reduced on day 3 post infection compared with the control mice. A lower degree of infection was observed with CR antibody. However,

while control animals showed a normal clearance of pulmonary virus 9-12 days after infection (11, 17) an unexpected finding was that the SSⁿ antibody treated animals showed a reduced clearance rate with virus being detectable on post infection day 12. In addition virus showing evidence of minor antigenic variation could be isolated from these animals. In another report (4) we present evidence that both SS and CR antibody preparations will select antigenic variants in the murine system but that the SSⁿ antibodies do so more efficiently than CR antibodies.

Little is known about the characteristics of serum anti HA antibodies which are most effective in conferring protection against influenza in man but our present results may have a bearing on the human situation. Our findings in mice imply that solid resistance to infection might best be provided by antibodies to the S antigenic determinants of the HA molecule of the infecting virus. However this type of antibody might be of more limited value in the natural protection against influenza in man because of the frequent antigenic drift in the HA antigens of the prevalent viruses. High levels of CR antibody might also confer some protection and against a wider range of antigenic variants. Once infection is established the SS and CR antibodies appear to interfere with viral replication to very similar degrees. CR antibodies may be more effective in this situation because of their broad reactivity with variant influenza viruses within a subtype and because they appear to be less effective in selecting new antigenic variants.

It has been shown (10, 14) that inactivated influenza A (H₃N₂) vaccines used in antigenically primed human adults in the interpandemic period may stimulate mainly CR antibodies and SS antibodies directed against the first member of the H₃N₂ subtype (A/HK/68 virus) rather than against the vaccine virus. In contrast in previously unprimed children who received similar vaccine SS antibody against the vaccine virus was frequently induced (Oxford *et al.* in preparation). Taken together with information on the differences in the biological activities of the CR and SS antibodies presented here these findings may be relevant to the understanding of the often incomplete and variable degrees of protection associated with the use of inactivated influenza vaccines in man.

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haemagglutinin (HA) antigen from HK/68 virus and various subfractions of anti-HA populations of antibody were prepared as described previously (5). In brief, the goat serum was exhaustively absorbed with concentrates of H3N2 variants A/England/42/72 and A/Victoria/3/75 in order to obtain different preparations of strain specific anti-HK/68 antibodies (SS' and SS, respectively) and cross-reactive antibody preparations (CR'' and CR', respectively). Post-infection ferret sera were prepared as described earlier (4).

Mice

Adult outbred female mice (NMRI/Bom), weighing between 20–22 g, were used.

Isolation of Variants

In allantois-on-shell The methods described previously were followed (4), except that antibody was added to the growth medium in a concentration 3–5 times as high as that required to inhibit growth of 500–1000 AOS infective doses of virus in 50% of the cultures.

In mice The IgG-dose of each antibody preparation required to inhibit death in 50% of mice challenged with four mouse lethal-doses (50%) of virus, 4 MLD₅₀, was determined as described before (6). In experiments where we looked for antigenic variants of HK/68 virus in mouse lungs, animals were given five times this IgG amount intraperitoneally and challenged intranasally with 1 MLD₅₀ of virus on the following day. For each

obtained at the dilution end-point was subjected to another egg passage at limiting dilution before being tested serologically. Also strains isolated in the AOS system were cloned twice in eggs before use.

Serological Tests

Haemagglutinations-inhibition (HI) tests and single radial haemolysis (SRH) absorption tests were done as

described in (13) and (8) respectively. Briefly, the SRH absorption assays were done as follows. SRH plate containing HK/68 virus or antigenic variants, were prepared. Three 20 µl aliquots of post infection ferret serum were mixed with equal volumes of either homologous, heterologous or control virus concentrate the control virus being A/chicken/Germany/N/12 (Hav2Neql), and left at room temperature for 1 h followed by 1 h at 4 °C, before testing for residual antibody in both homologous and heterologous SR plates. Lytic zones were measured by use of a calibrated viewer (Diffusion Zone Reader, Dynatech, Switzerland).

RESULTS

Apparent Frequency of Isolation of Antigenic Variants

In the course of this study it became evident that the various anti-HK/68 HA antibody preparation differed in their ability to select new antigenic variants. This is illustrated in Table 1. Both in the AOS system and in mice the most narrowly reacting SS antibody preparation (SS') exhibited the highest apparent ability to select new strains showing evidence of 'antigenic drift' in the HA antigen. However, the criterion for being an antigenic variant was that the isolated strains should react ≥ 2 -fold lower than the HK/68 virus in HI tests with the parent post-infection ferret serum. The occurrence of so-called 'non-avid' strains giving low HI titres in general (1), will obviously also score as an 'antigenic variant' in Table 1. These data should therefore be considered as describing the relative frequencies of appearance of new antigenic variants. For the *in vitro* and *in vivo* experiments taken together, the SS' antibodies seemed to provide the most successful growth advantage for antigenic

TABLE 1. Frequency of Isolation of HK/68 Variants giving ≥ 2 fold Reduction of HI Titres in Tests with Post-infection Ferret Serum to HK/68 Virus in Parallel Assays with Parent Virus

Selecting antibody ^{a)}	Frequency of variants		
	<i>In vitro</i> (AOS)	<i>In vivo</i> (mice)	Total
SS'	14/40 ^{b)}	2/25 ^{c)}	16/65 (25%)
CR'	5/33	3/24	8/57 (14%)
SS	11/34	14/25	25/59 (42%)
CR''	14/49	9/25	23/74 (31%)

a) SS' (SS) represents residual (strain specific) antibodies after absorption with the heterologous H3N2 strains A/Victoria/3/75 (A/England/42/72), whereas CR (CR') represents cross reactive antibodies eluted from the absorbing strains.

b) Ratio of number of strains being possible antigenic mutants to number of virus-positive AOS cultures.

c) Ratio of number of strains being possible antigenic mutants to number of virus positive mouse lungs (pairs) investigated.

TABLE 2 *Antigenic Relationships between A/Hong Kong/1/68 (H3N2) and Variants Obtained in Vitro and in Vivo in HI Tests Performed with Post-infection Ferret Sera*

<i>In vivo</i> variants						<i>In vitro</i> variants					
Virus strains	Antiserum to					Virus strains	Antiserum to				
	HK/68	SS 4B	CR 4E	SS 3E	CR 3D		HK/68	SS 4	CR 1	SS 22	CR 11
HK/68	10.32 ¹³	9.61	9.32	8.82	9.32	HK/68	10.12	9.61	9.32	9.61	8.32
SS 4B	9.32	10.11	9.61	8.82	8.91	SS 4	8.82	9.61	8.82	9.11	7.32
CR 4E	8.82	9.32	10.32	9.82	7.61	CR 1	8.32	8.32	9.32	8.61	9.11
SS 3E	9.11	7.82	8.82	9.61	9.32	SS 22	8.61	8.61	8.82	9.82	8.91
CR 3D	8.32	6.82	6.82	8.82	9.11	CR 11	9.32	8.11	8.11	8.32	10.11

¹ Log₁₀ geometric mean HI titre of duplicate tests

mutants the frequency being about 3 fold higher for the SS than for the CR antibodies. The corresponding frequencies for the two other antibody preparations, the more broadly reacting SS and the less cross reactive CR antibodies were of intermediate magnitude.

For the experiments in mice we also found that out of a total of 28 variants showing reduced HI titres with post-infection ferret serum to HK/68 virus, 20 were isolated on days 3 and 4 post-infection. Only SS-treated animals gave influenza isolates being possible antigenic mutants on day 5 post-infection.

Antigenic Relationship between Variants and Prototype Virus

HI tests. In Table 2 are presented HI data for one set of representative antigenic mutants obtained for each antibody preparation in tests with post-infection ferret sera. For sera against mutants

obtained both *in vitro* and *in vivo* there was a general tendency to give higher HI titres with the homologous than with the heterologous strains. Furthermore, by comparing the HI data for HK/68 virus and each mutant in tests with their respective sera, the HK/68 serum usually distinguished more clearly between parent and mutant strain than did each mutant serum. This is known as an asymmetrical reaction pattern (1). None of the mutant sera exhibited reaction patterns resembling that of the HK/68 serum, thus indicating that these isolates were in fact antigenic variants of HK/68 virus and not merely non-avid strains. Although all HI titrations were done in parallel and in duplicate with all dilutions of sera made up in bulk before transfer to titration plates and with the use of the same suspension of erythrocytes, the HI method is a rather inaccurate method of measuring antigenic similarities between related strains.

SRH absorption tests. These assays were done in

TABLE 3 *Antigenic Relationship between A/Hong Kong/1/68 (H3N2) and Variants Obtained in Vitro and in Vivo in SRH Cross-absorption Tests with Post-infection Ferret Sera*

Variants tested	<i>In vivo</i> variants		Variants tested	<i>In vitro</i> variants	
	HK/68 serum absorbed with variant tested in HK/68 plate	variant serum absorbed with HK/68 tested in variant plate		HK/68 serum absorbed with variant tested in HK/68 plate	variant serum absorbed with HK/68 tested in variant plate
SS 4B	19 ¹	10	SS 4	7	26
CR 4E	29	11	CR 1	5	25
SS 3E	35	26	SS 22	9	16
CR 3D	21	3	CR 11	2	31

¹ Per cent.

¹ In comparison with zone area after absorption with control virus A/chicken/

order to provide a more precise picture of the antigenic relatedness between mutants and parent virus. Post-infection ferret sera were exhaustively absorbed with concentrates of either homologous, heterologous or control ('N') viruses before testing for residual antibodies in homologous and heterologous SRH plates. As all homologous absorptions were complete, i.e. no lytic zones could be detected, only the results of the heterologous absorptions are shown in Table 3 and presented as per cent of lytic zone area remaining in comparison with area of control zone. Only infrequently were anti-neuraminidase antibody zones observed around the control wells, but their larger and weaker appearance made them easily distinguishable from the clearer and sharper anti HA zones. For the *in vivo* mutants it was demonstrated that a significant proportion of antibodies to the HA antigen could not be absorbed by heterologous virus. There was also a tendency for the mutants to absorb the anti-HK/68 antibodies less completely than in the *vice versa* type of experiments. Similar patterns of cross reactivity were seen for the *in vitro* mutants, although for these strains the anti-HK/68 antibodies were almost completely absorbed by the mutant strains.

DISCUSSION

We have described which specificity of antibody to the HA antigen of A/Hong Kong/1/68 (H3N2) virus, obtained from immune goat serum to purified HA antigen, seemed to have the property of selecting antigenic mutants by allowing the parent virus to multiply in the presence of either SS or CR antibody preparations. Our results clearly indicate that all antibody preparations tested have this property both *in vitro* (AOS) and *in vivo* (mice). The most narrowly reacting SS antibody preparation (SS₁) showed the highest capacity to select variants showing antigenic drift in the HA antigen whereas the most broadly reacting type of antibody (CR) provided less favourable conditions in this respect. However, the true capacity to select new antigenic variants cannot be resolved without preparing antisera to all possible antigenic variants. Assuming, however, that the occurrence of non avid strains was equally frequent for all antibody preparations tested, the overall data presented in Table 1 indicate that the SS₁ antibodies are the most effective for selective growth of new antigenic variants.

On the basis of our previous data (6), suggesting that the SS antibodies exhibit a higher neutralization capacity per molecule of IgG, both in the AOS system and in experimentally infected mice, it was

not unexpected that the specific antibodies seemed provide the most successful selective growth conditions for new antigenic variants. This has also been suggested by Virelizier *et al* (10).

However, all antibody preparations tested had the ability to provide selective growth advantages for new antigenic variants with changes in the HA antigen. The data presented in Table 2 showed that the various strains tested were different from the parent HK/68 virus based on the finding that antiserum to the parent virus reacted differently with the mutants than did the various mutant sera. This difference in antigenic profile between parent and each mutant may seem trivial, but this is to be expected when single-cycle mutants are tested (Haahm & Schild (4)). Furthermore, the cross absorptions experiments in SRH plates also showed that the reciprocal ability to absorb heterologous antibodies was not complete, confirming that an antigenic difference existed between the parent and each mutant (Table 3). Both for the *in vitro* and *in vivo* variants there was an asymmetrical pattern of absorption of heterologous antibodies, although the two groups of antigenic variants differed in this respect. However, the two methods of selecting antigenic mutants varied considerably in experimental design so that a straightforward comparison between observations may not be justified. Even so it is difficult to provide a reasonable explanation for this discrepancy.

The apparent frequency of isolation of variants seemed high for all antibody preparations tested. In view of recent works by Yendell *et al* (14) and Lubeck *et al* (7) suggesting that the frequency of isolation of antigenic variants employing mouse monoclonal antibodies to non overlapping antigenic sites on the HA antigen is in the order of 10^{-3} our data with polyclonal antibody preparations seem to be in conflict with theirs. Irrespective of how well defined the specificity of our goat antibody preparations may appear they probably comprise a range of antibody clones with varying specificities and affinities. It has been estimated that the probability of obtaining a double mutant, i.e. a variant with changes at two different antigenic loci by the use of two different monoclonal antibody populations in one single experiment should be in the order of about 10^{-10} per infectious unit of virus (12-14). Not surprisingly, such single step double mutants have not been isolated experimentally using high concentrations of monoclonal antibodies in the AOS system (14). However in our study we have used relatively dilute and carefully balanced concentrations of antibody. Consequently, only the most abundant clones or clone, of antibody would be expected to contribute to the selection process. Also

me of these antibodies were directed against overlapping antigenic sites the frequency of isolation of antigenic variants will not be the product of the abilities of mutations at each of the sites gauged by these antibodies.

We have in another study Oxford *et al* (in preparation) found that children have more SS antibody to current influenza strains than adults who by other hand have more CR antibodies as well as epidemiologically irrelevant SS antibodies to various variants within the HA antigenic subtype. SS antibodies of young children may therefore readily provide the best selective growth conditions for new antigenic variants. Furthermore we have recently tested some post-epidemic human sera against an antigenic mutant of the latest epidemic H3N2 variant A/Texas/1/77 obtained by passage of virus in the AOS system in the presence of a mouse monoclonal anti HA (Texas) antibody preparation (3). Not unexpectedly we found that sera from young children reacted to lower HI titres with the mutant virus than did sera from adults. These observations for human sera supplement the findings of the present report. Our main conclusion therefore is that the SS antibodies to the HA antigen will provide the best selective conditions for growth of antigenic variants.

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BRIEF REPORT

DEOXYRIBONUCLEIC ACID IS A SIGNIFICANT COMPONENT OF THE SMALL INTESTINAL MUCUS

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Ferencz A, Orskov I, Orskov F & Klemm P. Deoxyribonucleic acid is a significant component of the small intestinal mucus. *Acta path. microbiol. scand. Sect. B* 88: 347-348 1980.

During investigations into the role of intestinal mucus for the attachment of *Enterobacteriaceae* to the intestinal surface it was surprisingly found that DNA was a significant component of the mucus from the rabbit small intestine.

Key words: Small intestinal mucus, DNA.

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The attachment of bacteria to cells and mucous layers of human and animal origin has been studied in our laboratory (9-10). As part of this series of experiments we initiated investigations of attachment of bacteria to mucus from the small intestine of rabbits. Although many investigators stress the importance of the role of the small intestinal mucus for intestinal colonization (5, 6, 11) its chemical composition has not been well examined. In the following we describe highly purified DNA as a significant part of this material. Five rabbits weighing 3.5-4.0 kg were sacrificed. The small intestines were taken out and placed on ice. They were cut into pieces of 15 cm and the mucus was gently pressed out, gathered in a beaker on ice and kept refrigerated and sealed in mucus. No obvious differences between these preparations could be found. The mucus has a strong tendency to adhere to glass and polyethylene. The mucus was diluted into 12 volumes of ice-cold phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) and 0.1% EDTA.

After centrifugation of the suspended mucus (830 g for 10 min) a very compact pellet was formed which had approximately the same volume as the initial undiluted mucus. A thin layer of cells and bacteria was removed from the surface of the pellet by washing. The main part of the pellet consisting of a network of fibres could be stained with toluidine blue at pH 7.4 which is known to stain not only glycoproteins but also DNA. The lowest part of the pellet contained food particles and other materials such as sand and straw. These particles were attached firmly to the network of fibres in the pellet. Like the undiluted mucus the pellet was difficult to handle because of its extreme viscosity and centrifugation of the homogenate at higher g values only resulted in a compression of the pellet, making it even more difficult to handle.

The pellet was washed 5-10 times with ice-cold PBS and dissolved by addition of 4-6 volumes of 0.33 M NH₄OH. This procedure resulted in an extremely viscous solution from which the food remnants and other particles could not be removed.

Precipitation of the dissolved material could be done in two ways: by addition of PCA to a final concentration of 0.3 M or by addition of 3 volumes of 96% ethanol. The latter treatment resulted in the rapid appearance of long fibres which immediately aggregated to form a

TABLE 1 DNA RNA and Hexosamine Content ($\mu\text{g}/\text{mg}$ Protein) of the Small Intestinal Mucosa and Mucus in Rabbits

	Small intestinal mucosa ^a	Small intestinal mucus ^b	
		Supernatant	Pellet
DNA ^c	8.0	24.0	519.4
RNA ^d	54.8	40.0	24.0
Hexosamines ^e	3.8	1.9	4.3

^aMucosa from the wall of the small intestine was obtained by the method of Forsner (4). The mucosal suspension was precipitated by 0.3 M PCA and washed twice with 0.3 M PCA, extracted twice with 96% ethanol once with ethanol/ether 1:1 and twice with ether. The extracted sediment was dried at 37°C and dissolved in 0.5 M NaOH for further processing.

^bTo 3 ml of intestinal mucus 36 ml of PBS containing 100 $\mu\text{g}/\text{ml}$ TI was added. Homogenization was done by repeated pipetting with a wide tipped pipette. The suspension was centrifuged at 830 g for 10 min at 4°C. The supernatant was collected and PCA was added in aliquots to a final concentration of 0.3 M. Further processing was as described above for small intestinal mucosa. The pellet was washed 8 times with PBS and dissolved in 8 ml 0.33 M NH_4OH and 3 volumes of 96% ethanol were added. The resulting fibres were extracted with ethanol/ether and ether as above. The extracted fibres are designated 'pellet'. 1 mg of the dried material was dissolved in 1 ml of 0.5 M NaOH for further processing.

^cDNA content was estimated by the diphenylamine reaction (2) and a spectrophotometric method (12). These two methods gave identical results within the limits of the experimental error.

^dRNA content was estimated according to Flick & Begg (3).

^eHexosamine content of the hydrolysed samples was determined with a Durrum D 500 amino acid analyser as described earlier (8). Quantification was made relative to glucosamine.

^fThe protein content was estimated by the method of Itzhaki & Gill (7).

Bovine serum albumin was used as a standard and the results were corrected for TI content in the small intestinal mucus supernatant.

All operations were carried out at 0°C. Standard deviation of the results did not exceed $\pm 2\%$.

clump that entrapped the above mentioned particles. The DNA, RNA, protein and hexosamine content of these fibres and of the supernatant collected after the first centrifugation of the mucus was examined and compared with the content found in the mucosa of the small intestinal wall (Table 1). While the values in the three columns do not differ much from each other as regards

RNA and hexosamine, the high amount of DNA in the pellet was unexpected and remarkable. The nature of the ethanol precipitated material was examined by various methods. Resistance to alkaline hydrolysis, sensitivity to hydrolysis with 5 M PCA at 70°C/min as measured by increase in absorption at 260 nm and a positive diphenylamine reaction of the hydrolysate all indicated the presence of DNA. Furthermore the substance was sensitive to crystallization by pancreatic DNase I in the presence of Mg^{2+} ions.

In repeated experiments 56–88% of the DNA found in the pellet after the first centrifugation was difficult to make bacterial adhesion experiments with this highly insoluble DNA material. It has been possible to draw any conclusion about the nature of fimbriated or non fimbriated *Escherichia coli* DNA rich mucus.

We do not know the origin of DNA in the intestinal mucus. An alimentary origin of such amount of DNA is unlikely as is the bacterial because of the very few bacteria in the mucus. A tempting explanation is that the DNA originates from intestinal mucosal cells which are disrupted after release from the villi and then forms a new supporting the other components of the small intestinal mucus. Careful morphological and autoradiographic studies by Abrams *et al.* (1) of the small intestinal mucosal cell DNA labelled with tritiated thymidine shown that the turnover of the mucosal cells in the villi is a rapid process and that the movement of the generated cells progresses rapidly from the crypts to the luminal ends of the villi. However, the authors give data on the fate of the labelled DNA after release from the villi.

We believe that the demonstration of a considerable amount of DNA as a part of the small intestinal mucus is important for our understanding of the role played by small intestinal mucus in health and disease.

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BRIEF REPORT

COMPARISON OF THE SENSITIVITY OF ELISA AND THE HAEMAGGLUTINATION INHIBITION TEST FOR ROUTINE DIAGNOSIS OF RUBELLA

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Veytorp M & Leerhoy J Comparison of the sensitivity of ELISA and the haemagglutination inhibition test for routine diagnosis of rubella Acta path microbiol scand Sect B 88 349-350 1980

A total of 500 paired sera were tested for rubella antibodies by the rubella IgG ELISA and the haemagglutination inhibition (HI) test. Significant differences between the antibody level in the first and second samples of 417 serum pairs were not detected by either of the tests, whereas significant increases in 71 pairs were detected by both methods. A significant increase of the level in 12 serum pairs was demonstrated by the ELISA but not by the HI test. The rubella IgG ELISA is more sensitive than the HI test for diagnosis of recent rubella.

Key words: Rubella, routine diagnosis, ELISA, HI.

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A major problem in the serodiagnosis of recent infection by the haemagglutination inhibition (HI) test is the increase of specific antibody titers following the infection. Thus, the detection of a diagnostic increase of HI titer may be missed if the first sample is collected a few days after the rash. In a previous study of sera from a small number of patients a slower increase was noted when the rubella specific IgG antibodies were measured by the enzyme linked immuno-sorbent assay (ELISA). It was therefore presumed that the diagnostic sensitivity, i.e. the reliability of a negative test result (7), might be increased if the HI test was replaced by this test (6). A comparison of the two methods applied for the diagnosis of rubella is reported in the present communication.

Materials and Methods

A total of 500 paired sera submitted for routine diagnosis from patients with rashes exposed to or vaccinated against rubella were tested. The sera were given during a period of 3 months from general practitioners and hospital departments who also provided the clinical information. The sera were tested by the test and the rubella IgG ELISA as previously described (2, 3, 4). Each test had a control antigen was not

used in the ELISA, and the results were read on a Titertek Multiskan® photometer. The reported E values are the means of double determinations of single serum dilutions. The test was repeated if the difference between the two determinations of one serum exceeded 15%. An E value of 0.20 was used as the lower limit for the presence of antibodies.

Differences between the antibody levels in the paired sera were considered significant if the HI titer increased four fold or more or if the E values increased 20% or more provided the difference between the two values was more than 0.05 and antibodies were detected in the second sample. If only one of the sera was positive for rubella, the results were described as (6).

Results

Significant differences between the levels of rubella specific antibodies in the first and second of 417 of the 500 pairs of sera were not detected either by the HI test or the IgG ELISA. In 324 of these pairs rubella antibodies were detected by both methods, whereas 84 did not contain specific antibodies. Rubella IgG was demonstrated in 7 paired sera by ELISA but not by the HI test, the E values being 0.20-0.35 (median 0.31). The

HI titer of one pair of sera containing rubella IgG could not be determined owing to natural agglutinins still present after the pre treatment. The E values of one pair of sera with an HI titer of 10 were 0.17 and 0.18 respectively.

The coefficient of variation (standard deviation/mean) calculated from the difference between the first and second of the 417 samples was 17% of E values less than 0.20, 7% of E values between 0.21 and 0.50 and 4.5% of E values higher than 0.50.

Significant increases of the rubella antibody levels between the first and second of 71 paired sera were detected by ELISA and the HI test. The rise of the rubella IgG E values were 0.17–0.59 (median 0.35) corresponding to increases of 103–1680% (median 540) of the first value. The increases of the HI titers were 2–8 (median 5) of two fold titration steps. In each of 56 paired sera the first sample was collected 2 days after a rash or earlier, whereas the first sample of 2/3 and 2 paired sera were collected on days 3, 4 and 5 respectively. One pair was received from a patient exposed to rubella but without a rash, and one pair was collected before and after vaccination against rubella.

A significant increase of the rubella antibody concentration from the first to the second of 12 paired sera was demonstrated exclusively by the ELISA. Of these 11 pairs were collected from patients with rashes (Fig. 1).

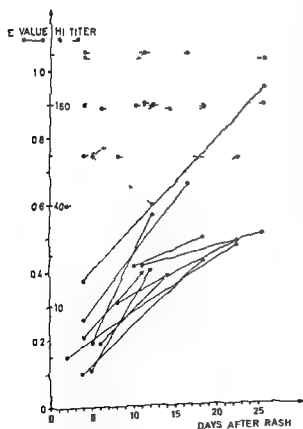


Fig. 1. Results of testing 11 paired sera in which a significant increase in concentration of specific antibodies was detected by rubella IgG ELISA but not by the HI test.

The increases of the E values were 21–333% (n = 156). All samples contained rubella specific antibodies detected by ELISA except one obtained days after a rash. Two sera which originated from patient before and after rubella vaccination contained rubella IgG in both samples (E values 0.25 and 1.0) but not rubella IgM antibodies.

Discussion

The discrepancy between the HI test and the rubella IgG ELISA was most marked in tests of sera of which the first had been obtained more than a few days after a rash. Thus 10 of 11 paired sera showing significant increases of the rubella IgG E values but not of the titers were collected 4 days after a rash or later (Fig. 1) as opposed to only 5 of the 63 sera in which significant increases were detected by both techniques. The cause of the different results of the two methods is therefore probably the rapid development of the antibody titers compared to the rubella IgG E values after rubella infections. This early increase in the titers is probably due to the contribution of specific antibodies which are not detected by the rubella ELISA. The previously demonstrated higher precision of the ELISA technique (4) may further contribute to the difference of the results.

An alternative method for serodiagnosis of rubella is the detection of specific IgM antibodies by ELISA. The assay may be applied routinely but is more complex than the IgG assay owing to the interference of IgM rheumatoid factor which necessitates determination of the concentration or removal of this anti-IgG by absorption to aggregated IgG (5). A new ELISA technique which uses anti-human IgM attached to a solid phase may be less affected by the presence of rheumatoid factor (1).

In a previous comparison of the ELISA and the HI test for detection of rubella antibodies the high sensitivity, the ease of performance and possibilities of automation of ELISA were emphasized (4). The present study also showed a higher diagnostic sensitivity of rubella IgG ELISA.

This study was supported by a grant from the Foundation of the Danish Health Insurance Society. Excellent technical assistance of Miss Karin Høiseth is gratefully acknowledged.

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BRIEF REPORT

HAEMAGGLUTINATION INHIBITION ANTIBODIES IN HUMAN SERA TO AN ANTIGENIC MUTANT OF INFLUENZA A/TEXAS/1/77 (H3N2) VIRUS OBTAINED *IN VITRO*

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Haasheim L. R. Haemagglutination inhibition antibodies in human sera to an antigenic mutant of influenza A/Texas/1/77 (H3N2) virus obtained *in vitro* Acta path microbiol scand Sect B 88 351-353 1980

An antigenic mutant of the wild influenza strain A/Texas/1/77 (H3N2) was obtained *in vitro* by growing virus in the allantois-on shell system in the presence of a mouse monoclonal antibody preparation to the Texas haemagglutinin (HA) antigen. A total of 204 human sera from a population of randomly collected sera during the fall of 1979 were examined by the haemagglutination inhibition (HI) tests against the Texas wild strain and the antigenic mutant. It was shown that sera from young children (0-5 years) reacted poorly or not at all with this mutant strain whereas sera from adults (15 years+) reacted equally well with both viruses. It can be assumed that the antigenic site recognised by this particular monoclonal antibody preparation (185/1) may be important for the human immune response to the HA antigen of A/Texas/1/77 virus.

Key words: influenza, antigenic mutant, monoclonal antibody, human sera.

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Monoclonal antibodies against the haemagglutinin (HA) antigen of various influenza viruses produced by the hybrids between mouse myeloma cells and red spleen cell (7) are now available (6, 13, 15). The aim of the present study was to determine whether such sera would inhibit viral haemagglutination in the presence of the wild influenza strain A/Texas/1/77 (H3N2) and a antigenic mutant obtained *in vitro* by growing A/Texas virus in the allantois-on shell system in the presence of monoclonal antibody against HA antigen. In particular we wanted to see whether sera from various age groups differed in their reactions with these two virus strains.

Materials and Methods

Virus strains. The preparation and antigenic characterization of antigenic mutants of A/Texas/1/77 (H3N2) virus by the use of mouse monoclonal antibody to the HA antigen of A/Texas virus will be described elsewhere (Haasheim *et al.* unpublished). In brief, the virus was cultivated in the allantois-on shell (AOS)

(Mo12) obtained through AOS passage of A/Texas virus with monoclonal 185/1. Post infection ferret serum to A/Texas virus gave 8 fold lower haemagglutination inhibition titre with the mutant strain than with the homologous variant whereas antiserum to Mo12 reacted to similar titres with both strains. In contrast the monoclonal antibody preparation used in our selection experiments gave a haemagglutination inhibition titre <50 with the mutant virus while having a titre of about

1000 in embryonated hens eggs (8) so that the H2 neuraminidase antigens were replaced by the Neu1 antigenic subtype. The recombinants will be called Tex and R12 respectively.

Human sera and serological tests. Sera were obtained from the following sources:

1. Human sera (14) except that the assays were performed in microplates employing 50 µl volumes of each reactant. All sera in this study were among those having HI titres ≥ 10 against the A/Texas prototype virus.

TABLE 1 *Haemagglutination inhibition (HI) Reactions for Human Sera in Tests with A/Texas/1/77 (H3N2) and Antigenic Mutant Selected in Vitro*

Age groups (years)	No of sera tested	Virus strains	No of sera with stated HI titres			GMT ^a	Ratio GMT Tex/R12	No of sera (%) with ≥ 4 fold lower HI titre with R12 than with Tex
			<10	10-20	≥ 40			
0-5	30	Tex ^b	0	6	24	62.1	3.3	20 (67)
		R12 ^c	8	12	10	19.1		
6-14	49	Tex	0	22	27	29.3	2.1	14 (29)
		R12	15	22	12	14.1		
15-24	39	Tex	0	9	30	46.1	1.4	1 (3)
		R12	0	16	23	33.5		
25-59	46	Tex	0	16	30	34.9	1.2	1 (2)
		R12	1	22	23	29.2		
60+	40	Tex	0	13	27	38.0	1.1	5 (13)
		R12	5	16	19	33.2		

^a Geometric mean HI titre. Titres <10 were assigned the value of 5 when calculating GMT.

^b A/Texas/1/77 recombinant with Neq1 neuraminidase.

^c Mutant Mo12 recombinant with Neq1 neuraminidase.

Results and Discussion

In all 204 post epidemic human sera from all age groups were assayed in HI tests with Tex and R12 viruses. Table 1 summarizes the main results of our study. For sera obtained from children aged 0-5 years the geometric mean HI titres (GMT) in tests with the two strains differed markedly (3.3 fold lower for R12). Also sera from older children (6-14 years) showed a clear fall in GMT (2.1 fold) whereas sera from adults (15 years +) reacted almost equally well with both strains. Twenty out of 30 sera (67%) from the youngest age group had HI titres ≥ 4 fold lower with R12 than with Tex virus. Of the sera from adults only 7 out of 125 had this reaction pattern. Again sera from older children showed an intermediate pattern of reactions with 14 out of 49 sera having ≥ 4 fold lower HI titres with the mutant virus.

Further analyses revealed that the 8 out of 30 sera (27%) from young children which failed to react with the R12 mutant (HI titres <10) had a GMT of 25.9 against the Tex variant (not presented in Table 1). The corresponding figures for older children were 15/49 (31%) and 13/8 respectively. Only 6 out of 125 sera (5%) from adults did not give positive reactions with R12 having at the same time a GMT of 10.8 against Tex virus.

It has been shown that young children respond to initial infection or vaccination by producing predominantly strain specific (SS) antibodies to the HA antigen. Influenza virus primed adults however usually respond by making cross reactive (CR) antibodies being able to react with a range of viruses within the subtype as well as SS antibodies directed against the first encountered member of the subtype (9, 10, Oxford *et al.* 1971). Furthermore the SS antibodies have higher capacity to neutralize virus (5, 11) to inhibit virus haemagglutination (4) and to modify viral replication (5) suggesting that young children with SS antibodies could be a candidate group for the selection of new antigenic variants showing 'antigenic drift' away from the previous prevalent strain and causing influenza epidemics at frequent intervals. The findings presented here are consistent with the previously reported distribution of SS and CR antibodies among various age groups since young children in contrast to adults reacted to low HI titres with the mutant strain R12 having at the same time relatively high HI titres against the latest epidemic H3N2 variant. It is likely that the SS antibodies may provide the most successful immunological pressure necessary for the selective growth of mutant viruses. We have in another study (3) demonstrated that SS antibodies from hyperimmune goat serum were highly

in selecting new antigenic variants both *in vitro* and *in vivo* (mice). This special role of SS was also suggested by *Virelizier et al* (12). The present report makes it likely that sera from children could provide at least theoretically the favourable growth conditions for new antigenic variants. Several passages in individuals, each having a different and restricted anti-HA antibody repertoire may be necessary in order to select a variant sufficiently antigenically different from previous strains to become immunologically relevant as has been suggested by *He et al* (15) and having the potential of spreading to groups other than the very young. Our mutant, assumed to have mutated at one antigenic locus coding for the antigenic site recognised by the monoclonal 185/1, may not have such an epidemic potential since adults had comparable levels of antibody to both the wild Type A/Texas and the antigenic mutant.

It is surprising that our mutant, having undergone several passages with only one monoclonal antibody, should react so poorly with children's sera. It is therefore assumed that the antigenic site used by monoclonal 185/1 could be an important site in the primary human immune response to A/WSN/77 virus. Similar studies are now in progress with other anti-Texas HA monoclonal antibodies in order to investigate whether changes at other antigenic sites were detected by human sera.

Supply of mouse monoclonal antibody 185/1 by Dr T G Webster, St Jude Children's Hospital, Mem-

phis, Tenn. and helpful discussions with Dr Geoffrey C Schild, National Institute for Biological Standards and Control, London, are gratefully acknowledged. Excellent technical assistance was provided by Paul H Burg.

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CONTENTS

Vol 88B Fasc 6 1980

Quantitation of M antigen in Lancefield extracts of group A streptococci type 12 using electro immuno assay <i>Ingvar Eliasson Maj Lis Svensson Goran Ramstorp Claes Schalén and Poul Christensen</i>	299
<i>Yersinia enterocolitica</i> and <i>Y. enterocolitica</i> like bacteria isolated from healthy humans in Norway <i>Georg Kapperud</i>	303
<i>In vitro</i> antibiotic sensitivity testing of <i>Vibrio alginolyticus</i> <i>Jens L. Larsen and Adel F. Farid</i>	307
An <i>in vitro</i> evaluation of two commercial blood culture media - supplemented peptone broth and supplemented tryptone soya broth <i>Per Sandven and E. Arne Høiby</i>	311
Resistance types in <i>Escherichia coli</i> II Transfer of ampicillin resistance <i>Per Sogaard</i>	317
Purification and characterization of aleutian disease virus <i>Bent Aasted</i>	323
Characteristics of antisera against periodate resistant membrane antigens from <i>Neisseria gonorrhoeae</i> <i>Sissel F. Røe Guri Eggset Ole Jan Iversen and Johan A. Mæland</i>	329
Antibodies to the strain specific and cross reactive determinants of the haemagglutinin of influenza H3N2 viruses 2 Antiviral activities of the antibodies in biological systems <i>L. R. Haaheim and G. C. Schild</i>	335
Antibodies to the strain specific and cross reactive determinants of the haemagglutinin of influenza H3N2 viruses 3 Selection of antigenic variants <i>in vitro</i> and <i>in vivo</i> <i>Lars R. Haaheim</i>	341

Brief Reports

Deoxyribonucleic acid is a significant component of the small intestinal mucus <i>A. Ferencz J. Ørskov F. Ørskov and P. Klemm</i>	347
Comparison of the sensitivity of ELISA and the haemagglutination inhibition test for routine diagnosis of rubella <i>M. Vejtorp and J. Leerhoy</i>	349
Haemagglutination inhibition antibodies in human sera to an antigenic mutant of influenza A/Texas/11/77 (H3N2) virus obtained <i>in vitro</i> <i>Lars R. Haaheim</i>	351

